GENETICS AND SYNTHESIS OF SUBUNITS OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE IN MEDICAGO

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DECLARATION

All the work reported in this thesis was done by myself except where expressly acknowledged. Specific mention should be made of analytical tasks kindly performed at my request by the following:

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List of Abbreviations

ATP	=	adenosine triphosphate
CSIRO	=	Commonwealth Scientific and Industrial Research Organization
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediaminetetra-acetic acid, disodium salt
HP	=	Hairy Peruvian cv. or Genotype
HR	=	Hunter River cv. or Genotype
MF	=	Medicago falcata
IEF	=	isoelectric focusing
kd	=	Kilodalton
LSU	=	large subunit
MBA	=	N,N'-Methylenebisacrylamide
ME	=	2-Mercaptoethanol
OD 260	=	optical absorbance at 260 nm
OD 280	=	optical absorbance at 280 nm
pI	=	pH of IEF
PIPES	=	piperazine-N,N'-bis(2-ethanesulfonic acid) monohydrate
RNA	=	ribonucleic acid
RuBPC-ase	=	ribulose 1,5-bisphosphate carboxylase
SDS	=	sodium dodecyl sulphate
SSU	=	small subunit
TEMED	=	N,N,N',N'-Tetramethylethylenediamine
TCA	=	trichloroacetic acid
TRIS	=	tris-hydroxymethyl aminomethane

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ABSTRACT

This thesis reports experiments which examine the phenotypic and genotypic properties of subunits of ribulose 1,5-bisphosphate carboxylase (RuBPC-ase) and the effects of temperature and light intensity on synthesis of subunits of RuBPC-ase in Medicago species.

The subunit composition of S-carboxymethylated RuBPC-ase was investigated in M. sativa and M. falcata by isoelectric focusing. Three large subunits (LSU) and two small subunits (SSU) were found in the above species. In contrast, isoelectric focusing of RuBPC-ase without S-carboxymethylation showed one LSU and two or three SSU polypeptides in M. sativa and M. falcata respectively. S-carboxymethylation affected the composition of both LSU and SSU polypeptides in Medicago. One LSU polypeptide was confirmed in Nicotiana and Spinacea as well. It seems likely that one LSU polypeptide is the rule in higher plants, instead of three LSU previously reported.

Analysis of amino acid composition of LSU and SSU polypeptides suggested a more conserved nature for the LSU than for the SSU polypeptide in Medicago species. The peptide maps and amino acid composition supported the results of isoelectric focusing, which showed differences in the number of bands and pI values of SSU polypeptides of the two Medicago species.

Studies of quantitative genetics of leaf proteins were carried out during three selection cycles. Response to selection for high protein content showed 75% heritability. Both RuBPC-ase and "cytoplasmic' protein increased or decreased simultaneously during the selection. The high and low protein contents were correlated with chloroplast area. Although the LSU polypeptide is under chloroplast gene

control, the experimental evidence indicated that the quantitative changes of RuBPC-ase are under nuclear gene control.

Plants of M. sativa and M. falcata were grown at 25/19°C and 35/30°C day/night temperatures. Plants of the above two species were also grown under low light intensity conditions for one to four days. The synthesis of LSU and SSU polypeptides was investigated under these conditions. Results showed that high temperature and low light intensity reduced the synthesis of both LSU and SSU polypeptides, but the reduction was greater in the case of the LSU than for the SSU polypeptides, indicating an uncoupling of co-ordinated synthesis under stress conditions. The Hairy Peruvian (HP) genotype was found to be more sensitive to extreme environmental stress conditions than was M. falcata, as shown also by a significant genotype/environment interaction.

The genetics and evolution of RuBPC-ase in higher plants are discussed in terms of a mutation hypothesis and a multigene structure/function hypothesis.

CHAPTER 1.

INTRODUCTION

1.1. GENERAL INTRODUCTION

The term "Fraction I protein" was introduced by Wildman and Bonner in 1947 to designate a high molecular weight protein which occurred in leaves. This protein comprises up to 50% of the soluble protein of leaves and is probably the most abundant protein in nature (Kawashima and Wildman, 1970a). Fraction I protein is responsible for catalysing the initial step of photosynthesis and that of ribulose bisphosphate oxygenase activity. Weissbach et al., (1956) succeeded in purifying the enzyme responsible for the first step in the Calvin photosynthetic cycle, the carboxylation of ribulose bisphosphate with $C0_2$ to form 3-phosphoglyceric acid. It became apparent that the two activities, carboxylation and oxygenation, were in fact performed by the same enzyme. Hence RuBPC-ase is responsible for catalyzing the initial step of photosynthesis, the process on which all life, as we know it, depends.

1.2. STRUCTURE OF RuBPC-ase

All Fraction I proteins (ribulose bisphosphate carboxylase : RuBPC-ase) so far isolated from higher plants have a sedimentation constant of about 18 S, corresponding to a molecular weight of 5.6×10^5 daltons (Kawashima and Wildman, 1970a; Kung, 1976). Treatment with sodium dodecylsulphate results in the dissociation of RuBPC-ase into subunits of two sizes, a large subunit (LSU) of 56,000 daltons and a small subunit (SSU) of 12,000 daltons in Nicotiana (Kung et al., 1974). In an extensive study of crystalline RuBPC-ase from

tobacco Baker et al. (1975, 1977), using X-ray diffraction, electron microscopy and optical diffraction, concluded that the holoprotein was made up of eight LSU and eight SSU pairs. It has been demonstrated (Kung et al., 1974; Chen et al., 1976) that when RuBPC-ase protein from a variety of plants is examined by isoelectric focusing, the LSU of each plant can be separated into three distinct polypeptides, while the SSU yields from one to four different polypeptides. Interspecific comparison showed that the particular set of LSU and SSU could vary from one species to another. A comparison of the amino acids of LSU and SSU from a number of plants revealed that the amino acid composition of the LSU from higher plants (Kawashima and Wildman, 1970a; Sugiyama and Akazawa, 1970; Kawashima et al., 1971; Strobaek and Gibbons 1976), as well as from Chlorella ellipsoides (Sugiyama et al., 1971) and Euglena gracilis (Rabinowitz et al., 1975), was very similar, whereas the SSU varied widely in the amino acid composition. This similarity in the LSU from different species is also apparent from their immunological properties, since antisera to LSU from different genera exhibit cross reactivity to one another, whereas the SSU antisera show no common determinants with either SSU from other species or LSU from the same species (Kawashima and Wildman, 1971b; Kawashima et al., 1971; Gray and Kekwick, 1974; Brown et al. 1976).

In contrast, O'Connell and Brady (1981) demonstrated that RuBPC-ase consists of one LSU and one type of SSU in Triticum aestivum. They found that carbamidomethylation of the enzyme before isoelectric focusing resulted in three bands of LSU.

It has been suggested that the lack of variability of the LSU results from the cistrons being in a polyploid condition and therefore there is little selection for mutational changes (Kirk, 1972). The SSU, which is coded for by nuclear DNA is, as expected, more variable since this DNA is more responsive to mutational forces. Chen et al., (1976) calculated that the cistron coding for the large RuBPC-ase subunits of Nicotiana spp. from Australia has not had one surviving mutation in 10^8 years, whereas the SSU has a mutation rate similar to that of cytochrome c. However, examination of the tryptic peptide maps of four species of Nicotiana has led to the conclusion that the mutation rate is, in fact, higher than that predicted on the basis of the isoelectric points (Kung and Lee, 1977). While the structure of RuBPC-ase from higher plants, and from green and most blue-green algae (Rabinowitz et al., 1975) conform to the above generalization regarding subunit variability, RuBPC-ases from photosynthetic bacteria appear more variable. For instance, those from Rhodospirillum rubum, Rhodopseudomonas spp., and Chlorobium spp. are significantly smaller molecules (Anderson et al., 1968; Tabita and McFadden, 1972; Tabita et al., 1974) while the enzyme from Hydrogenomonas, a chemoautotroph, was originally reported to consist of just one subunit of 40,000 daltons (McFadden, 1973). Recently Purohit and McFadden (1976) have shown that there are two LSU of 52,000 daltons and 56,000 daltons occurring in a molar ratio of 3:5, as well as one SSU of 15,000 daltons. Gibson and Tabita (1977) have also isolated two forms of RuBPC-ase from Rhodopseudomonas sphaeroides. One form from Rhodospirillum and Chlorobium contains only the LSU of about 54,000 daltons; the other form resembles higher plant enzymes by containing both a LSU and a SSU. The

RuBPC-ase from the blue-green alga Agmenellum quadriplicatum of 456,000 daltons consists of only one subunit which has a M.W. of 56,000 daltons (Tabita et al., 1974).

Enzymes with oxygenase activity often contain co-factors such as copper, iron and flavin nucleotides (Hayaishi, 1974; Pistorius and Axelrod, 1974). A report that RuBPC-ase from spinach contained 1 g atom of bound copper per mole of enzyme (Wishnick et al., 1969, 1970) was queried by Lorimer et al., (1973), who found less than 0.2 g-atom per mole. Chollet et al., (1975) have reported the absence of bound copper, iron or flavin nucleotide in crystalline RuBPC-ase from tobacco.

1.3. ISOLATION OF RuBPC-ase

The most widely used procedures for the isolation and purification of RuBPC-ase include deionization of crude leaf extracts by gel filtration followed by purification on Sephadex G200 or Sepharose 6B and subsequent ion exchange chromatography with DEAE cellulose (Trown, 1965; Steer et al., 1968; Sugiyama, et al., 1968; Kawashima, 1969; Kawashima and Wildman, 1971a; Haslett et al., 1976; Strobaek and Gibbons, 1976). Alternatively, density gradient centrifugation on gradients of sorbitol (Kleinkopf et al., 1970) or sucrose (Goldthwaite and Bogorad, 1971; Givan and Criddle, 1972; McFadden et al., 1975; Takabe et al., 1976) takes advantage of the high molecular weight (560,000 daltons) of RuBPC-ase to achieve considerable purification. An elegant method for the preparation and recrystallization of pure RuBPC-ase from Nicotiana spp. has been described (Chan et al., 1972). This method has been adapted to the isolation of large quantities of RuBPC-ase from tobacco by Lowe (1977).

RuBPC-ase oxygenase was isolated and crystallized from eight plant species. Crystals grew from either of two similar sets of crystallizing conditions: crystals of the enzyme from lucerne, corn, cotton, potato, spinach, tobacco, and tomato grew from solutions containing phosphate and polyethylene glycol 6000 as a precipitant; those from potato, tobacco (both Nicotiana sylvestris and Nicotiana tabacum), and tomato grew from a mixture of ammonium sulphate and phosphate. Crystals of the enzyme from potato and both species of tobacco were large enough to characterize by X-ray diffraction and were found to have the Form III structure, previously reported for crystals of RuBPC-ase from N. tabacum. For crystalline material from several species, both carboxylase and oxygenase activities have been asssayed and copper and iron contents have been determined (Johal et al., 1980).

A rapid separation technique, developed by O'Connell and Brady (1981), consists in isolation of RuBPC-ase by polyacrylamide gel electrophoresis, followed by a tube electrophoresis of the homogenised gel containing RuBPC-ase.

The subunits are readily separated by gel-filtration on Sephadex G100 in the presence of urea (Moon and Thomson, 1969), sodium dodecyl sulphate (Rutner and Lane, 1967; Gray and Kekwick, 1974) or alkali (Kawashima and Wildman, 1970b). Gray and Wildman (1976) have described a method of isolation of the mixed subunits by use of a column containing RuBPC-ase antibodies linked covalently to Sepharose 4B, in which the crude extracts are passed through the column and the mixture of pure subunits eluted by dissociation of the antigen-antibody complexes with 8 M urea.

1.4. PHYSIOLOGY OF RuBPC-ase

Investigations concerning some physiological properties of RuBPC-ase included oxygen dependent deactivation and reactivation, stability under various conditions, and effect of hardening. RuBPC-ase of spinach is deactivated by the removal of oxygen and reversibly reactivated by addition of oxygen to the enzyme solution. A short pulse of oxygen to the anaerobic enzyme solution is sufficient to trigger the activation process; the K_a value for the reaction was estimated as 0.12 mM oxygen. The enzyme could not be reactivated under anaerobic conditions by an organic oxidant (benzoylperoxide) or by sulphydryl-group reducing reagents (dithiothreitol or beta-mercaptoethanol), suggesting that the reactivation process was oxygen specific. Furthermore the inhibition of the reactivation by superoxide anion scavengers such as Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid), copper, penicillamine, hydroxylamine, nitroblue tetrazolium, and ascorbate indicated that the monovalent reduced oxygen was involved as a reacting species in this process. The deactivation of the enzyme associated with the removal of oxygen was also sensitive to the presence of scavengers of O_2^-, suggesting that superoxide anion was also involved in the deactivation process. Both the carboxylase and the oxygenase activities were similarly affected under all the experimental conditions studied. On the basis of these results it is argued that the enzyme molecules are able to reduce oxygen and that superoxide anions cause the deactivation or reactivation of the enzyme (Henkel et al., 1980).

Activation of RuBPC-ase by oxygen in spinach was demonstrated by Wildner et al., (1980). Both activities, deactivation by removal of oxygen and reversible reactivation by oxygenation of the enzyme

solution were demonstrated. The change in enzyme activities was accompanied by conformational changes as studied by the use of intrinsic and extrinsic fluorescence probes. The analysis of cysteine sulphydryl groups accessible to 5,5'-dithiobis-(2-nitrobenzoic acid) revealed that the number of these groups changed with oxygen concentration. The kinetics of the exposure of eight cysteine residues parallelled the loss of enzyme activities. The modification of these groups with 5,5'-dithiobis-(2-nitrobenzoic acid) caused almost complete loss of both activities. The enzyme isolated from a photo-lithotrophic organism, Chromatium vinosum, was not affected by oxygen removal. During the air-argon transitions, neither the enzyme conformation nor the number of accessible sulphydryl groups changed.

The specific activity of crystallized Nicotiana RuBPC-ase did not change during 12 days' storage at room temperature. When placed in an ice bath, 70% of the activity disappeared within 24 hours and remained lost during continued storage at 0°C. However, the lost activity could be completely regained at any time during cold storage by a 20 min treatment at 50°C. Loss or restoration of activity was not accompanied by a detectable change in the sedimentation velocity properties of RuBPC-ase (Kawashima et al., 1971).

Change in the net charge and subunit properties of RuBPC-ase during cold hardening was demonstrated by Huner and MacDowall (1979). RuBPC-ase from leaves of cold-hardened and unhardened Puma rye was purified by gel filtration and ion-exchange chromatography. The specific activity of the hardened form was twice that of the unhardened form. A difference in charge between the two forms of this enzyme was shown by gel electrofocusing. The

estimated isoelectric point (pI) values were 6.4 and 6.3 for the enzyme from the hardened and unhardened source, respectively. The LSU (55,000 molecular weight) of the enzyme from one unhardened source formed an apparent dimer during sodium dodecyl sulphate gel electrophoresis. At pH 6.8 it was also the source of an anomalous polypeptide with an apparent M.W. of 47,000, which appeared in both hardened and unhardened preparations after irreversible inactivation of RuBPC-ase activity by NaCl. This polypeptide also appeared after preparation of the purified enzymes for SDS polyacrylamide gel electrophoresis in the absence of beta-mercaptoethanol, but this was reversible. Evidence was obtained for protein changes being involved in protection against freeze inactivation by cold hardening of the enzyme. A freeze-thaw cycle applied to the enzyme in vitro caused some polymerization of the LSU and its anomalous polypeptide in the absence of reducing agent, especially with unhardened preparations. This effect increased with repeated cycles until the fifth cycle when the LSU monomer and its satellite were abolished, but only in preparations from the unhardened source. These data indicate that the LSU is the probable site of a change occurring in this enzyme during cold hardening.

A comparison of structure with function was made with RuBPC-ase from leaves of non-acclimated, cold-hardy Solanum commersonii (Sc) and non-hardy S. tuberosum (St) (Huner et al., 1980). The kinetics of SH titration with 5,5-dithiobis (2-nitrobenzoic acid) and the number of accessible SH groups in the native enzymes were different but the total titratable SH/mole RuBPC-ase was the same. The native molecular weight was 544,000 for both enzymes. SDS had a differential effect on the kinetics of SH titration. The

quaternary structure of both enzymes was the same with the presence of LSU (54,000 M.W.) and SSU (14,000 M.W.). However, the LSU of St RuBPC-ase had a greater propensity for dimerization, and was more sensitive to the absence of reducing agent and to freeze-thawing than the LSU of Sc RuBPC-ase. No structural changes were observed for the SSU from either species. The catalytic properties of both enzymes at 5° and 25°C indicated no significant difference in $K_m CO_2$ at either temperature. However, V_{max} for St RuBPC-ase was 25% greater at 25°C than that of Sc RuBPC-ase, but was 35% lower at 5°C.

Quaternary structures of RuBPC-ase from cold-hardened and unhardened Puma rye were examined by two-dimensional gel electrophoresis (Huner and Hayden, 1982). The results indicate that major changes in charge heterogeneity occur in the LSU of this enzyme during growth at cold-hardening temperatures. The extent of charge heterogeneity decreased upon adaptation of Puma rye to cold-hardening temperatures. In addition to charge heterogeneity, molecular weight heterogeneity was also evident in the LSU polypeptides of the enzyme from cold-hardened and unhardened Puma rye.

Patterns of seasonal variation of enzyme levels in the brown alga Laminaria hyperborea (Gunn.) Fose. have been investigated (Küppers and Weidner, 1980) for RuBPC-ase. This enzyme exhibit a circannual periodicity, characterized by a pronounced "spring-maximum" of enzyme activity in April and May. As a consequence the phylloid can maintain high metabolic rates from early spring on, although the water temperature has then only slightly risen above the annual minimum. The seasonal patterns outlined above correlate well with

the circannual fluctuation of the nitrogen content of the sea and with variation in the internal nitrogen and nitrate content of the alga. This coincidence may indicate that nitrogen levels play an important role in the regulation of enzyme activities, and hence, the metabolic capacities of L. hyperborea.

1.5. SITE OF SYNTHESIS OF RUBPC-ase

Since it was established around 1965 that chloroplasts contain all necessary components for autonomy, namely DNA, DNA-dependent RNA polymerase, and the complete machinery for protein synthesis (Kirk and Tilney-Bassett, 1967) it appeared possible that chloroplast DNA contained the information to code for all chloroplast constituents and that chloroplasts could transcribe the DNA and translate this information into the unique enzymes and structural proteins which were required for organelle development and for the functioning of the photosynthetic apparatus. The three main approaches to the question of whether RuBPC-ase is synthesised within the chloroplast on the 70S ribosomal system or on the 80S ribosomal system of the cytoplasm have been (1) in vivo use of inhibitors which differentially inhibit the two translating systems, (2) the use of isolated chloroplast preparations to make specific polypeptides in the absence of cytoplasmic factors, and (3) isolation of chloroplast polyribosomes and immunoprecipitation of either the nascent polypeptides attached to the polyribosomes or in vitro products of translation from these polysomes.

1.5.1. Inhibitor Studies

Most authors using Euglena (Davis and Merrett, 1975), Chlamydomonas (Margulies, 1971; Armstrong, et al., 1971; Iwanij et

al., 1975), or higher plants (Graham et al., 1970; Criddle et al., 1970; Owens and Bruening, 1975; Beisenherz and Koth, 1975) have reported that RuBPC-ase activity is decreased both by inhibitors such as chloramphenicol, which affect the 70S chloroplast protein synthesising system, as well as those such as cycloheximide, which inhibit the 80S cytoplasmic system. Most inhibitors of the two systems did not always affect both systems equally and most observations are consistent with the theory that the LSU of RuBPC-ase is synthesised in the chloroplast and the SSU in the cytoplasm, and that there is a degree of coupling or coordination of the synthesis of the two. The two observations that do not fit with this conclusion are the two cases where cycloheximide was found to stimulate the synthesis of RuBPC-ase (Smillie et al., 1971; Givan, 1974).

1.5.2. Isolated Chloroplast System

Harris et al., (1973) using Euglena gracilis, found that the radioactive protein synthesised by isolated chloroplasts coincided with RuBPC-ase on a gel column. Ramirez et al., (1968) observed that chloroplasts of spinach, when isolated in a medium using KCl as osmoticum, incorporated labeled amino acids into the protein fraction, using light to generate the necessary ATP via endogenous photophosphorylation. The products of this reaction in pea chloroplasts were examined by Blair and Ellis (1973) who found the sole soluble radioactive product coincided with the LSU of RuBPC-ase on SDS-polyacrylamide gels. Its identity with the LSU was confirmed by peptide mapping. Bottomley et al. (1974) found that similar light-driven amino acid incorporation occurred in spinach chloroplasts which had been isolated in a sorbitol medium. It has been shown

(Vasconcelos, 1976) that intact chloroplasts of Euglena, purified by centrifugation on silica gel gradients, also made the LSU of RuBPC-ase as the major soluble product when incubated in the light. It is clear from this result with isolated chloroplasts that the mRNA for the LSU is contained in chloroplasts and that the chloroplast protein-synthesising system has the capacity to synthesise this polypeptide.

1.5.3. Immuno Precipitation

Assembly of newly-synthesised LSU into RuBPC-ase in isolated intact pea chloroplasts was demonstrated by Barraclough and Ellis (1980). Isolated pea (Pisum sativum) chloroplasts incorporated ^{35}S-methionine into the LSU of the chloroplast enzyme, RuBPC-ase. The chloroplasts were incubated in a medium containing KCl as osmoticum; newly-synthesised LSU was not incorporated into the holoenzyme but could be separated from pre-existing enzyme by gel electrophoresis under non-denaturating conditions. Furthermore, newly-synthesised LSU's were not precipitated by antibodies which precipitated pre-existing holoenzyme and LSU prepared from holoenzyme. When chloroplasts were incubated in a medium containing sorbitol as osmoticum, some of the newly-synthesised LSU's co-migrated with holoenzyme on both 3% and 5% polyacrylamide non-denaturing gels. Such co-migrating LSU's were precipitated by antibodies raised against the holoenzyme. These results indicated assembly of LSU's into RuBPC-ase in the sorbitol medium. Time course experiments showed a time-lag of several minutes between onset of synthesis of LSU's and the onset of assembly. Newly-synthesised LSU's which did not co-migrate with holoenzyme on both 3% and 5% polyacrylamide non-denatured gels were associated

with a protein of subunit molecular weight 60,000. This protein could be specifically combined with newly-synthesised LSU's, and the resulting aggregate was involved in the assembly of complete molecules of RuBPC-ase.

The technique of immunoprecipitation is a potentially powerful tool for testing for a single species of polypeptide in a heterogeneous mixture. Several workers have used this procedure to approach the question of the site of synthesis of the RuBPC-ase subunits. The general approach has been to isolate either 70S or 80S polyribosomes from plants actively synthesising RuBPC-ase, and to determine whether either the nascent peptides on the polyribosomes or the polypeptide chains completed and released in vitro are precipitated by the antiserum made against the LSU or the SSU of RuBPC-ase. In 1973 Gooding et al. isolated both membrane-bound and free ribosomes from wheat seedlings and separated the 70S and 80S size classes by sucrose gradients. When the nascent peptides were released by treatment with ^{3}H-puromycin it was found that antibodies to the LSU of RuBPC-ase precipitated peptides only from free 70S ribosomes, whereas both anti-small subunit and anti-large-subunit antibodies precipitated peptides from the membrane-bound 80S ribosomes.

1.6. LOCATION OF STRUCTURAL GENES AND ASSEMBLY OF THE SUBUNITS

It is clear that the LSU of RuBPC-ase is synthesised in the chloroplast on 70S ribosomes, whereas the SSU is synthesised in the cytoplasm on 80S ribosomes as a precursor of higher molecular weight than the small subunit itself.

There is no direct evidence as yet as to the site or mechanism of assembly of the two subunits into an active RuBPC-ase complex. The observation of Gooding et al. (1973) that in wheat seedlings the SSU appears to be synthesised on membrane-bound cytoplasmic polysomes led them to suggest that this membrane can at some time become attached to, or part of, the chloroplast outer membrane and so allow the SSU to pass into the chloroplast either during synthesis or following dissociation from the polyribosomes. This suggestion was supported by the observation of Cobb and Wellburn (1976) that a protein with the properties of the SSU of RuBPC-ase became associated with the chloroplast envelope fraction of Avena sativa during the early greening process. That the chloroplast is the site of assembly of the two subunits is supported by recent work which indicates that the SSU is synthesised in the cytoplasm as a precursor of about 20,000 daltons. This precursor has been observed in the product from cell-free synthesis on polyribosomes of wheat by Roy et al. (1976), as well as in the translation products of cytoplasmic mRNA of Chlamydomonas (Dobberstein et al. 1977) and peas (Cashmore et al. 1978; Highfield and Ellis 1978). In addition, precursor has been shown to be processed to the SSU by an endoprotease associated with polysomes (Dobberstein et al., 1977) or by intact chloroplasts (Highfield and Ellis, 1978). The latter authors also found that when the precursor was processed by intact chloroplasts the SSU was resistant to protease digestion and concluded that it must have passed through the outer membrane into the chloroplast. This strongly suggests that the site of assembly of the subunits must be within the chloroplast envelope.

It has been postulated by Ellis et al. (1978) that protein synthesis within the chloroplast is controlled by the synthesis of nuclear coded polypeptides. This theory is supported by the observation of Feierabend and Wildner (1978) that the SSU accumulates in rye leaves at 32°C, where chloroplast protein synthesis is inhibited due to a deficiency of 70S ribosomes. On the other hand Hallier et al. (1978) have shown that in RuBPC-ase-deficient mutants of Oenothera, where the deficiency is caused by a mutation in the plastid DNA, no SSU could be detected, suggesting that the synthesis of the SSU is being controlled by the synthesis of the LSU. It seems probable, therefore, that the subunits of RuBPC-ase protein are assembled within the plastid but the mechanism controlling the synthesis and assembly is, as yet, not understood.

1.7. GENETICS AND EVOLUTION OF RuBPC-ase

The sites of the cistrons coding for both the LSU and SSU of RuBPC-ase have been established by Wildman and his collaborators (Chan and Wildman, 1972; Kawashima and Wildman, 1972). These experiments take advantage of the relative ease with which interspecific hybridization within the genus Nicotiana takes place, and also of the fact that the DNA from chloroplasts of this and many other species is only inherited through the maternal parent (cytoplasmic inheritance), whereas the nuclear genome is inherited from both parents (Mendelian inheritance). The first method used by these authors was to resolve differences in the tryptic peptide composition of the subunits of different species, and to follow the inheritance patterns of these peptides. It was found (Kawashima and Wildman, 1972) from peptide maps of SSU that Nicotiana tabacum

exhibited one peptide that was absent in N. glutinosa and two that were absent from N. glauca. When reciprocal crosses were made using N. tabacum alternatively as the male or female parent, it was found that these peptides appeared in the F_1 progeny when N. tabacum was either the male or female parent, showing that the genetic information could be transmitted via the pollen. This indicated that the cistrons for SSU of RuBPC-ase are in the nuclear DNA.

The LSU exhibits considerably less variability in amino acid composition than the small subunit (Kawashima and Wildman, 1970a). No differences were found in the tryptic peptide maps of the LSU of any Nicotiana spp. indigenous to the Western hemisphere but it was discovered that species from Australia contained one peptide not present in N. tabacum. Reciprocal crosses demonstrated that this peptide was present in hybrids only when an Australian species was the maternal parent, hence the genetic information for the large subunit must be contained in the chloroplast DNA (Chan and Wildman, 1972).

These results have been supported by an examination of the mode of inheritance of differences in polypeptide composition revealed by isoelectric focusing of the S-carboxymethylated LSU and SSU of interspecific hybrids of Nicotiana spp. Again it was shown that the information for the composition of the SSU is inherited in a Mendelian manner, whereas that for the LSU is inherited maternally (Kung et al., 1974).

Direct evidence that chloroplast DNA contains the LSU cistron has been obtained by the cell-free transcription and translation of a cloned fragment of corn chloroplast DNA (Coen et al., 1977) and also

of total spinach chloroplast DNA (Bottomley and Whitfeld, 1979). It seems clear now that the LSU of RuBPC-ase is coded for by chloroplast DNA and synthesised by the 70S ribosomal system in the chloroplast, whereas the SSU is coded for by the nuclear DNA and synthesised on 80S ribosomes in the cytoplasm. The major problem remaining on the mechanism of synthesis of this protein is the means by which the synthesis of these two types of subunits is coordinated and how they are assembled into the active enzyme.

The structural gene for the LSU of RuBPC-ase in Zea mays was shown to be contained within a 2500 base pair sequence of chloroplast DNA. One copy of this DNA sequence is present in each maize chloroplast circular DNA molecule. It maps approximately 30,000 base pairs from the 5' end of the closest of two sets of rRNA genes and approximately 71,000 base pairs from the other set of rRNA genes (Bedbrook et al., 1979).

Progeny analysis of interspecific somatic hybrids was carried out by Aviv et al. (1980). The progeny of a fusion experiment involving N. sylvestris protoplasts and X-irradiated protoplasts of the cytoplasmic male sterile "Line 92" (N. tabacum nucleus and alien, male-sterility inducing cytoplasm) were analysed. Three groups of somatic hybrid plants resulted: Type A, Type B-1, and Type B-2. These as well as their androgenic progenies and the progenies resulting from their pollination with N. tabacum or N. sylvestris were followed with respect to several nuclear and cytoplasmic traits. Those controlled by the nuclear genome were plant and flower morphologies; those controlled by genetic information in the cytoplasm were tentoxin sensitivity, the LSU of RuBPC-ase, and the restriction endonuclease pattern of plastid DNA. A further cytoplasmic trait

investigated was male sterility. The examination of the somatic-hybrid groups and their respective progenies indicated that Type A plants have N. sylvestris nuclei and "Line 92" plasmids; Type B-1 plants also have "line 92" plasmids, but their non-plastid genome is composed of N. sylvestris and N. tabacum nuclei; Type B-2 plants with impaired male fertility had N. sylvestris plastids and N. sylvestris nuclei.

1.8. QUANTITATIVE GENETICS

The majority of morphological and physiological characters in eukaryotes depend upon alleles at many loci, and they have been the subject of much investigation by quantitative analysis. Response to selection varies according to the magnitude of the genetic variance, more especially to the relative proportion of additive and non-additive gene effects. If the proportion of additive variance is high the rate of response to selection will be rapid; if the additive variance is low the expected response to selection will be moderate.

Response to selection for quantitative characters has been well studied in Drosophila, maize, and lucerne.

Selection for body weight in Drosophila melanogaster was carried out by Sheldon (1963). Two replicate mass selection lines were developed in both high and low directions for nearly 40 generations. Significant responses to both high and low selection occurred.

A further experiment concerned 135 generations of selection for high scutellar bristle number in Drosophila melanogaster. Two lines were derived from the same original mating of one female with 5 bristles by one male with 4 bristles. In a selection line "M", an early period of rapid response when the selection intensity was highest was

followed by a short plateau at 7.5 to 8 bristles for about 10 generations (Sheldon and Milton, 1972). Further selection for about 15 generations showed a moderate response, a similar period of minor "creeping" response, and a long period of about 30 generations at a second "plateau" level of 10 to 10.5 bristles. Finally a short burst over 5 generations lifted the mean by 2 to 2.5 bristles to a plateau level of 12-13 bristles.

The effects of selection on oil content in the grain of maize was reported by Alexander and Creech (1977). The selection for oil content was carried out for 15 generations and the population mean increased from 5% to over 15.3%. In selecting for protein content in maize populations the mean was increased to 26.6%, as reported by Dudley et al. (1974) in the Illinois High Protein Strain after 70 generations of selection, 12 standard deviations above the mean of the original population. The mean protein content of the Illinois Low Protein Strain was 4.4% after 70 generations of selection.

Temperature can markedly influence the expression of polygenes in populations of Medicago sativa. Under warm temperature conditions dry matter production is controlled by genes with both additive and non-additive effects, while under cool temperature conditions the character is controlled by genes showing additive effects only.

At warm temperatures non-additive gene effects normally prevail and therefore there is little response to both family and individual selection following growth at such temperatures. Despite this, it was possible, following extensive hybridization, to produce recombinant genotypes which also responded to selection at warm temperatures (Daday, 1965).

The response to selection after recombination of genotypes in lucerne was extensively investigated. Biometrical analysis has shown that cold hardiness in lucerne depends upon genes with additive effects which respond rapidly and predictably to selection (Greenham and Daday, 1957; Greenham and Daday, 1960).

Artificial selection causes dramatic changes in the composition of the genetic variance that contributes to the frequency of creeping root. These variances were largely additive between the second and third generations of selection. This additive effect was, however, reduced from 50-60% realised heritability to 10% in later generations, so that the genetic variance then consisted largely of non-additive gene effects. In consequence, mass and family selection, dependent on additive gene action, is relatively ineffective after the seventh generation. Heterosis, dependent on non-additive gene action, then assumes most significance in increasing the frequency of creeping root characters (Daday, 1962).

Significant genotype x locality and genotype x season interactions can be demonstrated in lucerne. These show that temperature is the major factor involved in the genotype-environment interaction (Daday et al., 1968) and suggest that the non-additive genetic component of yield is a temperature-dependent phenomenon.

Selection for any one of the three characters-plant diameter, plant survival and degree of creeping root, leads to concomitant changes in the other two. This suggests that the three characters comprise an adaptive complex which must be maintained in any breeding programme (Daday, 1968).

Despite the finding in other organisms that there is no depression of heritability even under extreme stress conditions

(Falconer, 1952; Falconer and Latyszewski, 1952), stresses on growth in lucerne lead to a significant reduction in heritability. Response to selection is thus markedly reduced under extreme stress conditions. Correspondingly, it appears to be safe to say that (at least for the feature investigated in the species studied) predicted selection-response is substantially increased by judicious choice of optimal (in some instances) or moderate stress conditions (in other instances) (Daday et al. 1973).

However, as shown also by earlier investigators, Daday et al., (1977) found decisive evidence (statistically significant) that a substantial fraction of the genetic determination of creeping-root in lucerne is due mainly to non-additive alleles. This phenomenon is important for more efficient genetic improvement of a character (economically valuable under certain conditions) in an important cultivar.

The above quantitative analyses are relevant to the present investigations in providing a guide for determining the type of genetic control of leaf protein synthesis, namely response to selection of RuBPC-ase and cytoplasmic proteins. It is also important to determine any correlation between changes in protein content and cell morphology. In addition, selection experiments may reveal whether the ratio of LSU to SSU can be altered as a result of genetic manipulation.

The major difference between the previously-used quantitative analyses and the present one is that the present investigation stresses the biochemical mechanism of selection, which was not a feature in the previous investigations.

1.9. CHLOROPLASTS OF HIGHER PLANTS

The chloroplasts of plants are generally considered to be semi-autonomous organelles which increase in number by division (Granick, 1961; Frey-Wyssling and Muhlethaler, 1965; Kirk and Tilney-Bassett, 1967).

Granick (1938) observed that during the growth of tomato leaves from one-third of maximum size to full size the number of plastids per cell increased by about 30%. Fasse-Fransisket (1955) found that during the differentiation of Agapanthus umbellatus leaves the numbers of plastids per cell increased from about 20 in meristematic cells to 100 to 120 in mature palisade cells. Using phase-contrast microscopy she observed frequent division by contriction of the amoeboid granular plastids in the leaf cells of this plant, but less frequent division of fully differentiated and somatic chloroplasts. Division of the young, "amoeboid" proplastids of the meristematic cells of Epilobium has also been observed by Michaelis (1962). In this plant, most of the plastid division took place in the early stages of chloroplast development. By contrast, Wildman (1967), who has made extensive light-microscope observations on living cells of Spinacea oleracea, has reported being unable to find any evidence of chloroplast division occurring in the mature cell of this plant.

Possingham and Saurer (1969) found that the amount of chlorophyll and nitrogen and the number of cells per unit area changed as the green leaves of spinach plants grew and increased in size in the light. A five-fold increase in the number of chloroplasts per cell took place in both palisade and mesophyll cells during a growing period of 10 days when the area of the leaves increased from 1 to 50 cm^2. Proplastids were not present in the young green leaves

but electron-microscope and phase-contrast observations showed the presence of grana-containing chloroplasts, many of which appeared to be undergoing division by constriction. They suggested that the large increase in chloroplast numbers as leaf cells grow and expand in light results from the division of differentiated chloroplasts containing grana.

Investigations concerning the factors affecting chloroplast replication in spinach were carried out by Possingham and Smith (1972). Replication was studied in discs cut from the base of young spinach leaves and cultured on sterile nutrient agar. In discs grown in a growth cabinet, chloroplast numbers per cell increased logarithmically with time over a 7-day culture period. Chloroplast replication proceeded in a similar way in cultured discs and in intact leaves. Cytokinins did not affect chloroplast replication in this system but they stimulated the fresh-weight growth of discs. Chloroplast replication was temperature dependent, having an optimum at 25°C. By contrast, chloroplast size was at a maximum in discs cultured at 12°C. Light stimulated chloroplast replication, a linear relationship occurring between chloroplast number per cell and the daily quantity of light given to discs up to a saturating value of 250 Jd^{-1}. Day length did not affect chloroplast formation in spinach. A general relationship was established between chloroplast number per cell and cell size but no evidence was available to suggest that this correlation was causal. The results of experiments in which discs were transferred from dark to light suggested that some of the events which precede chloroplast replication may occur at similar rates in both light and dark.

Chloroplast replication in spinach leaf discs is stimulated by white, blue or red light, with intensities of up to 5 mW cm^{-2} being required for saturation (Possingham and Smith, 1972; Possingham, 1973). The development and photosynthetic capacity of chloroplasts formed in green light in cultured spinach leaf discs has been investigated by Possingham et al. (1975). At intensities of 4 to 6 mW cm^{-2} green light stimulated chloroplast replication to about the same extent as white, blue, and red light. However, practically no chloroplast replication occurred in discs grown in low intensity green or white light, but considerable chloroplast growth took place. Ultrastructural studies have shown that these chloroplasts, which can be two to five times the area of control plastids (formed under high intensity white light) have an essentially normal thylakoid system. Double isotope labelling experiments have established that the synthesis of chloroplast ribosomal-RNA is similar in controls and in discs grown in low-intensity green or white light. On a per-unit chlorophyll basis the $C0_2$ fixation rate of spinach discs grown in low intensity green (or white) light saturates with increasing light intensity or increasing $C0_2$ concentration at values well below control discs. In this respect the photosynthetic characteristics bear a similarity to those of shade plants.

The above investigations showed developmental and environmental effects on chloroplast number and size. Molin et al. (1982) demonstrated that ploidy also affects chloroplast number in M. sativa L. Protoplasts obtained from tetraploid M. sativa contained twice as many chloroplasts per cell as the diploid M. sativa.

There is no published information available concerning the effects of continuous genetic selection on chloroplast sizes and their biochemical consequences.

1.10. EFFECT OF DEVELOPMENTAL AND ENVIRONMENTAL FACTORS ON THE SYNTHESIS OF RuBPC-ase

The level of RuBPC-ase of leaves is greatly influenced by the developmental stage of the plant and also by environmental factors.

Changes in genome expression during normal cellular development in the first leaf of young (7-day-old) wheat (Triticum aestivum var. Maris Dove) were investigated by examining homogeneous populations of leaf cells of several developmental ages, present in the same leaf (Dean and Leech, 1982). All of the leaf cells had plastid, organelles and nuclei, and between 44% (young tissue) and 54% (older tissue) of the leaf cells were mesophyll cells. Large changes occurred in cellular constituents over a very short period of leaf development. Maximum rates of accumulation of RuBPC-ase per mesophyll cell (80 pico-grams $hour^{-1}$) and of 70S ribosomes per mesophyll cell (19 x 10^5/hour) were recorded. The period of maximum accumulation of protein, total RNA and both 80S and 70S ribosomes occurred between 36 and 48 hours after the last cell division. Between 48 and 60 hours, 70S rRNA per cell and protein content per cell continued to increase as 80S rRNA per cell declined, and RuBPC-ase per cell increased 20-fold between 15 and 60 hours.

Stamp (1980) investigated the activity of RuBPC-ase in leaves of maize (Zea mays L.) seedlings in relation to temperature changes. Four inbred lines of flint maize were grown at 24/22°C (high) or 14/12°C (low) until the full expansion of the second leaf, when samples of these leaves were taken. Thereafter the temperature was changed from high to low or from low to high. One, two and five days later samples were taken again and leaves were assayed for RuBPC-ase content. Growth at low temperatures as compared with

high gave considerably higher genetic variability. A lower RuBPC-ase activity was found at low as compared to high temperature. Insignificant changes in RuBPC-ase activity were found after transition from high to low temperature. A rapid and steady increase in RuBPC-ase activity was demonstrated after transition from low to high temperature. RuBPC-ase could possibly be limiting in some genotypes at different temperatures.

Increase in level of RuBPC-ase as a result of light-induced increase of mRNA activity coding for the SSU of RuBPC-ase was demonstrated by Sasaki et al., (1981). The effect of light on the levels of mRNA coding for the SSU of RuBPC-ase was measured together with enzyme levels. The mRNA level, the enzyme level and the rate of enzyme and SSU synthesis were all increased by illumination. The increase in the rate of SSU synthesis was directly proportional to the increase in translatable mRNA coding for the SSU. These results indicate that the light-induced increase of RuBPC-ase is controlled by the mRNA level for the SSU which in turn is modulated by a nuclear-transcriptional process or by a light-evoked stabilization of mRNA. During induction the pool of mRNA increased with illumination time in proportion to the total amount of subunit synthesis. The rate of RuBPC-ase synthesis was proportional to the rate of SSU synthesis. These results imply that the induction, which requires the cooperation of nuclei and chloroplasts, is regulated by the SSU mRNA level rather than by a cytoplasmic translational process, transportation of the precursor, or assembly of the subunits.

The published research reviewed above clearly shows that developmental and environmental factors can affect the level of

synthesis of RuBPC-ase. Still further investigations are required to examine genotype x environmental interaction.

1.11. SCOPE OF THIS INVESTIGATION

The main aim of this investigation is to analyse the subunit structure, genetic control, and subunit synthesis of RuBPC-ase as affected by environmental factors (high temperature, low light intensity) in Medicago genotypes.

The subunit structure of RuBPC-ase of genotypes was compared by isoelectric focusing. Commercial equipment for isoelectric focusing is available, but a more efficient apparatus was developed as described in Chapter 2.

Subunits of RuBPC-ase of M. sativa were analysed by Chen et al., (1977), and their results are re-examined in Chapter 3. RuBPC-ase of three genotypes of Medicago were isolated by column chromatography, and the subunit structure was compared. The tryptic maps of LSU and SSU of two Medicago species were prepared in order to determine species-specific peptide differences in subunits.

There appears to be only a single LSU gene in plastid DNA coding for LSU of RuBPC-ase. Previous published work had indicated three LSU bands, while recently published results showed that rapid isolation of RuBPC-ase with reduced chemical pretreatment exhibits one single LSU band in wheat. Results of my investigations which support the general validity of one single LSU are based on analysis of several plant species as described in Chapter 4. The amino acid composition of LSU and SSU for the three genotypes of Medicago is also considered in the same chapter and the evolutionary significance of pI values of subunits and amino acid composition is discussed.

Analysis of quantitative characters helps to disclose the type of genetic determination and any correlation with other metric characters. Response to selection for high and low protein content was investigated for three generations and detailed studies of correlations between protein content and characters of relevant morphology are described in Chapter 5. This type of research may also disclose functional relationships between increased protein synthesis and the morphology of the cell.

Very little published work is available on the effect of environmental factors on the synthesis of subunits of RuBPC-ase. Therefore studies were undertaken on the effects of high temperature and low light intensity on the synthesis of LSU and SSU in two genotypes of Medicago, as summarized in Chapter 6.

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CHAPTER 2.

MATERIALS AND METHODS

2.1. PLANT MATERIALS

Plant materials utilized in various experiments were as follows:

Medicago sativa cv. Hunter River (HR), cv. Hairy Peruvian (HP), and cv. Du Puits (S313). All are commercial cultivars from South Australia.

M. sativa cv. Rambler (C.P.I. 30598) and M. falcata, Sc-1765 tetraploid (C.P.I. 96983). Both were obtained from the Canadian Department of Agriculture, Swift Current, Saskatchewan, Canada.

Nicotiana excelsior (7553), N. tabacum (Q 1232) and N. glutinosa (R 608-5) were provided by the Department of Primary Industries (Mareeba, Queensland).

Seed of spinach (Spinacea oleracea) was obtained from local commercial sources.

2.2. REAGENTS

Acrylamide (laboratory reagent grade) was supplied by BDH. Urea was recrystallized twice from 95% ethanol. Tris (hydroxymethyl) aminomethane was supplied by Calbiochem-Behring Corp. Cleland's reagent (dithiothreitol). A grade was obtained from Calbiochem. Ampholine, pH 6-8, was purchased from LKB. The calibration Kits, pH range 3-10, and the High-pI Calibration Kit, pH range 5-10.5 (including the marker molecules lentil lectin-basic, lentil lectin-middle, lentil lectin-acidic, myoglobin-basic, myoglobin acidic, and human carbonic anhydrase B), were obtained from Pharmacia. TEMED (N,N,N',N'-tetramethylethylene diamine) was from Sigma.

All the other reagents were standard laboratory chemicals.

2.3. CULTURE OF PLANTS

Plants of the above species were grown in 15-cm (using standard glasshouse soil) under natural light at 1200-1500 $\mu E.m^{-2}s^{-1}$, in a heated glasshouse with the temperature maintained between 20° and 25°C. Plants in the phytotron were grown in 15-cm pots (with perlite) and provided with Hoagland solution in the morning and water in the afternoon. These plants were kept either at 25/19°C or 35/30°C. The reduced day/light treatment was carried out at 12 $\mu E.m^{-2}s^{-1}$ for 12 h daily at 25/19°C during a period of one to four days. Samples of leaves were collected for protein extraction when the plants reached the green bud stage. The material used in our studies contained approximately 20% dry matter and 16.5% protein (dry matter basis).

2.4. COLUMN CHROMATOGRAPHY

2.4.1. Separation of Total Leaf Protein by G25 Column Chromatography

Three gm of fresh leaf material was collected and homogenized in the presence of 3 ml extraction buffer (Tris 200 mM, Na_2 EDTA 5 mM, $MgSO_4 . 7H_2O$ 25 mM, polyvinylpyrrolidone 1%, 2-mercaptoethanol 10 mM, cysteine 5 mM; adjusted to pH 8.5 with boric acid) in a precooled mortar, at 4°C. The resulting homogenate was squeezed through a layer of Miracloth. The filtrate was centrifuged at 108,000 g for 45 min. The supernatant was applied to a G25 column (1.5 x 30 cm). Fractionation was carried out at 4°C, using "running buffer" (Tris 45 mM, boric acid 25 mM, Na_2 EDTA 0.7 mM, pH 8.5). The flow rate was 0.1 ml min^{-1} and each eluted fraction was one ml.

2.4.2. Isolation of RuBPC-ase by Sepharose 6B Column Chromatography

Leaf samples (10 g) were frozen in liquid nitrogen and finely chopped in an Omnimix homogeniser. The chopped material was then shaken for 20 min in 20 ml of 25 mM Tris-HCl (pH 6.8) containing 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA and 40 mM 2-mercaptoethanol in an ice bath. The resulting homogenate was squeezed through two layers of Miracloth (Chicopee Mills Inc.). The filtrate was centrifuged at 20,000 g for 30 min and the supernatant was recentrifuged at 108,000 g for 75 min. Five ml of the resulting supernatant was applied to a Sepharose 6B column (1.5 x 86 cm). Fractionation was carried out at 4°C using 20 mM Tris-HCl, pH 6.8, containing 50 mM NaCl and 0.02% sodium azide. The flow rate was 0.1 ml min^{-1} and each eluted fraction was 2.5 ml. The fractions rich in RuBPC-ase were further purified by a modification of the technique of Kawashima and Wildman (1970). The sample was dialysed against one litre of 0.05 M Tris-HCl, pH 7.6, containing 0.01 M NaCl and 0.5 mM EDTA.

2.4.3. Separation of LSU and SSU by G100 Column Chromatography

Precipitated RuBPC-ase was dissolved in 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 percent sodium dodecylsulfate and was passed through a 2.5 cm x 85 cm Sephadex G100 column previously eqilibrated with the same buffer. Fractions 20-44 (1 ml each) were collected for assay of LSU and SSU.

2.4.4. DEAE-Cellulose (DE-52) Column Chromatography

A DEAE-cellulose (DE 52) column (1.5 x 20 cm) was equilibrated with 50 mM Tris-HCl buffer and eluted with a gradient of 0.1 to 0.5

M NaCl in the same buffer at 4°C. RuBPC-ase samples were applied to the column and 1 ml fractions were collected and monitored for protein content by measuring absorbance at 280 nm. The purified protein was used for gel electrophoretic studies.

2.5. GEL ELECTROPHORESIS

2.5.1. Isolation of RuBPC-ase by 6% Davis Gel Electrophoresis

Stock solutions for the Davis (1964) type of polyacrylamide gel were as follows:

A	I N HCL	48 ml	C	Acrylamide		45.0 gm
	TRIS	36.6 gm		MBA		1.2 gm
	TEMED	0.23 ml		H_2O	to	150 ml
	H_2O	to 100 ml				
	pH	8.9	D	Acrylamide		5 gm
				MBA		1.25 gm
B	I N HCL	24 ml		H_2O	to	50 ml
	TRIS	2.99 gm	E	Riboflavin		2 mg
	TEMED	0.23 ml		H_2O	to	50 ml
	H_2O	to 50 ml				
	pH	6.7	F	Sucrose		40 gm
				H_2O	to	100 ml

The glass frame to hold the polyacrylamide slab gel was prepared from two standard (8 x 8 cm) glass electrophoresis plates and two glass supporting rods which were glued together. The sides and bottom of the plates were sealed with adhesive tape prior to pouring of the gel.

Two separate solutions, a separating gel solution and a concentrating gel solution, were prepared.

"Separating" gel solution:		"Concentrating" gel solution:	
H_2O	16.2 ml	F	2 ml
A	3.0 ml	B	0.5 ml
C	4.8 ml	E	0.5 ml
Ammonium persulphate, dissolved in 0.5 ml of distilled water	17 mg	D	1 ml

The air was removed from both solutions under vacuum.

The glass frame was filled with the separating gel up to a height of 6 cm and kept at 4°C overnight. One cm layer of concentrating gel solution was added and exposed to fluorescent light for 30 min.

The gel was placed into an electrophoresis tank containing the following electrophoresis buffer : Tris 45 mM, boric acid 25 mM and Na_2- EDTA 0.7 mM, pH 8.5. A 0.7 ml plant extract sample (see Ch. 2.4.1.) was applied and the gel was run at 30 mA and approximately 120 V for 3 h.

2.5.2. SDS Gel Electrophoresis of Precipitated RuBPC-ase

Protein in the fractions from the Sepharose 6B column was precipitated with 10% (w/v) trichloracetic acid (TCA) at 4°C for two hours and centrifuged at 12,000 g for 10 min. The supernatant was discarded and the pellet washed twice with cold 10% TCA. The pellet was dissolved in 70 µl of a solution containing 0.15 g SDS, 0.8 g

sucrose, 100 µl 2-mercaptoethanol and 0.01 g bromophenol-blue per 10 ml of Tris-glycine buffer, pH 8.2, by heating in boiling water for 2 min.

Proteins were separated on 10% acrylamide SDS gels (Thomson and Schroeder 1978) using a Gradipore electrophoresis apparatus (Scientific and Research Equipment, Sydney). The gel buffer was 25 mM Tris (pH 8.2) and 200 mM glycine, while the upper tray contained the same buffer with 0.1% sodium dodecyl sulphate (SDS). The gels were pre-run for 2 h at approx. 150 V, 30 mA prior to sample application and then run at the same voltage and current for 1.5 h.

Proteins were stained with Coomassie blue (4 g Coomassie brilliant blue in one litre of absolute ethanol, 200 ml acetic acid and 800 ml water).

2.5.3. SDS Gel Electrophoresis of RuBPC-ase by 13% Davis Gel

The stock solutions of the Davis (1964) polyacrylamide gel technique (see Chapter 2.5.1.) were used to prepare the 13% Davis gel.

The glass frame was used as described in Chapter 2.5.1.

Composition of the 13% Davis gel :

	H_2O	10.6 ml
Solution	A	3.0 ml
Solution	C	10.4 ml
	SDS	24 mg
Ammonium persulphate, dissolved in 0.5 ml of distilled water		17 mg

The gel was prepared and kept at 4°C overnight.

One ml of a fraction rich in RuBPC-ase (4 mg protein) was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, and was loaded on a Davis (1964) slab gel. The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA and 0.1% SDS, pH 8.5. Electrophoresis was carried out for 2 h at 30 mA and approx. 120 V at room temperature. The edges of the gel were cut off and stained in Coomassie Blue. The LSU and SSU bands were located in the gel and the strips of gel containing the proteins were cut out. The gel strips were homogenised in the above buffer and placed in electrophoresis tubes. Tubes were covered at one end with cellophane membrane and the protein in the gel was recovered by electroelution (5 mA per tube) overnight.

2.5.4. Starch Gel Electrophoresis

Samples of protein from the second and third peaks eluted from Sepharose 6B columns were electrophoresed on starch gel and stained for a range of enzymes. Electrophoresis was conducted in a discontinuous buffer system (pH 8.0) using 13% starch gels (Shaw and Koen 1968). Running conditions were those described previously by Marshall and Allard (1970). At the completion of electrophoresis, each gel was cut horizontally into three slices and each slice was stained for a different enzyme. The staining procedures were similar to those described by Brewer and Sing (1970) and Brown et al. (1978).

2.5.5. SDS Tube Electrophoresis of LSU and SSU Polypeptides

The band of RuBPC-ase was cut out from the 6% gel slab and homogenised by passing through a 1 ml tuberculin syringe into a gel tube containing a polyethylene frit at the lower end. The gel tubes were connected by a short rubber cuff to glass thimbles sealed with a semipermeable membrane, and the whole apparatus filled with electrophoresis buffer (Tris 45 mM, boric acid 25 mM, Na_2 EDTA 0.7 mM, pH 8.5 and 5% SDS). After electrophoresis for 4 h at 1 mA per tube, the RuBPC-ase had moved from the gel pieces through the frit and was evident as a refractile zone adjacent to the semi-permeable membrane. The sample was removed from the thimble with a microsyringe and the amount of enzyme estimated by ultraviolet absorbance at 280 nm (A: 1.6 = 1 mg/ml). All operations were carried out at room temperature.

2.6. ASSAY OF RuBPC-ase ACTIVITY

Samples of the second and third peaks from the Sepharose 6B column were adjusted to pH 8 with 10 mM Tricine-KOH buffer containing 5 mM dithiothreitol and 10 mM $MgCl_2$ to give a final protein concentration of 0.1 mg ml^{-1}. The enzyme was activated by pretreatment with Mg^{++} and HCO_3^- and then assayed as described by Lorimer et al. (1976). Protein was determined by the procedure of Warburg and Christian (1941).

2.7. PROTEIN ASSAYS NON-PROTEIN NITROGEN ASSAYS AND AMINO ACID ANALYSIS

Protein was measured using the method of Lowry et al. (1951) and nitrogen determinations were done with a Kjeldahl automatic analyser (Williams and Twine 1967).

Non-protein nitrogen was assayed according to Freney et al. (1977). 500 mg ground lucerne was boiled in 10 ml of 70% ethanol for 10 min. The suspension was filtered through a Whatman No. 42 filter and washed several times with boiling 70% ethanol until 100 ml was collected. The ethanol was evaporated under vacuum. The filtrate contained the non-protein nitrogen which was determined by Kjeldahl automatic analysis (Williams and Twine, 1967).

Amino acid compositions were determined by the method of Byers (1971).

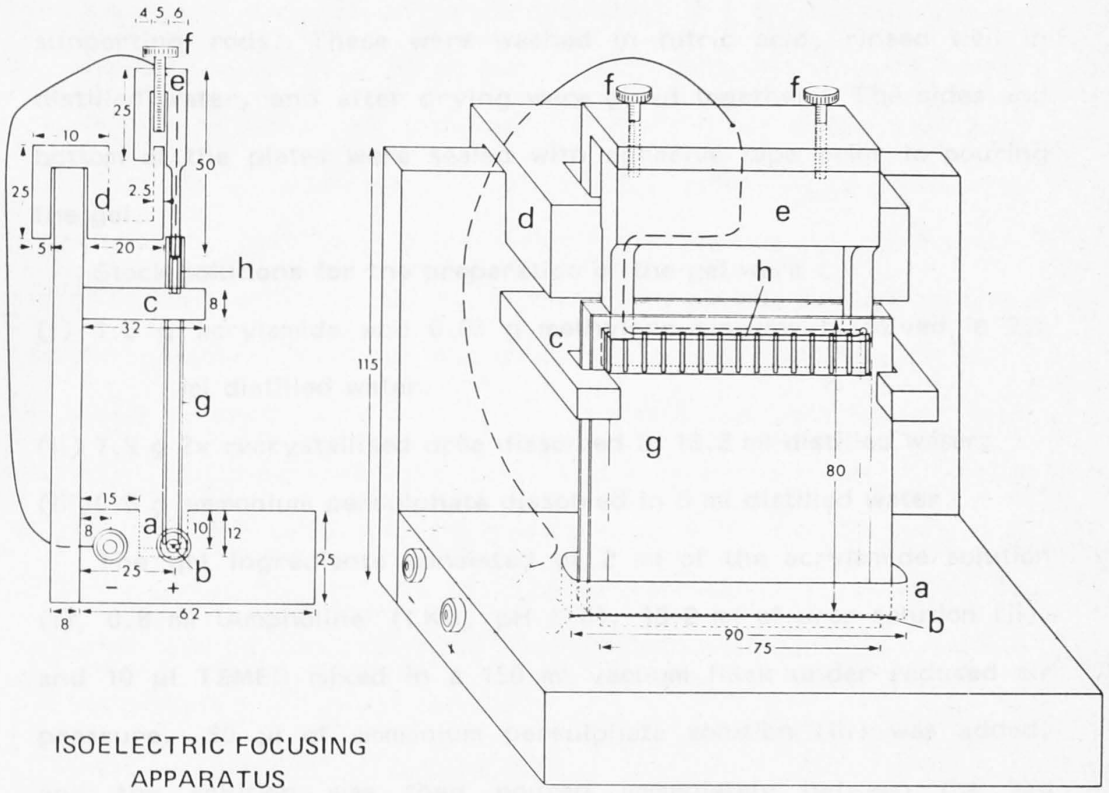
2.8. ISOELECTRIC FOCUSING

2.8.1. Description of the Apparatus

The apparatus (Fig. 2.1) was constructed from 25 mm and 8 mm (thickness) Perspex sheets. One buffer trough (a) was cut into the horizontal base plate. The two ends of this trough, which is 10 mm deep for most of its length, have a raised step of 2 mm above the floor of the trough, enabling buffer movement to proceed to the end of the gel. This modified apparatus is provided with a minimum amount of buffer in order to create an even pH gradient. The gel itself, sandwiched between glass plates (described in the next section), is held in position vertically by two holding brackets (c) attached to the rear vertical frame. Also bracketed to the rear vertical wall at the top is a holder featuring a U-section deep depression (d). This bracket, which is fixed, supports the frame (e) carrying the negative electrode (0.5 mm Pt wire), while adjusting screws (f) permit the variable positioning of the carrier frame and consequently the level of the negative electrode. The positive electrode is located in the lower trough (Daday and Whitecross, 1983).

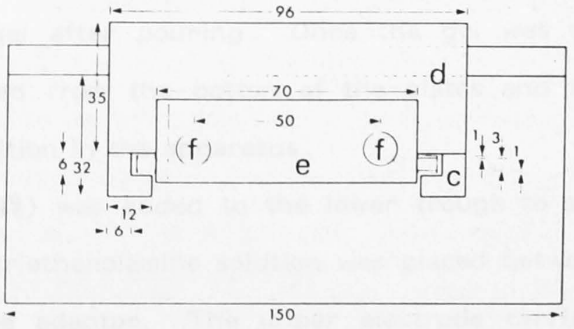
Fig. 2.1. Isoelectric focusing apparatus.

This modified apparatus consists of a base and horizontal and vertical plates. The gel itself, sandwiched between glass plates (g), is held in position vertically by two brackets (c) attached to the rear vertical frame. The frame (e) carries the negative electrode. One buffer trough (a) was cut into the horizontal base plate. The positive electrode (b) is located in the lower trough (a). The same adaptor (h) was placed on the gel between glass plates (g).



ISOELECTRIC FOCUSING APPARATUS

(All dimensions measured in millimetres)



2.8.2. Preparation of the Gel Slab

The glass frame for the polyacrylamide gel was prepared from two standard (8 x 8 cm) glass electrophoresis plates and two glass supporting rods. These were washed in nitric acid, rinsed well in distilled water, and after drying were glued together. The sides and bottom of the plates were sealed with adhesive tape prior to pouring the gel.

Stock solutions for the preparation of the gel were :

(i) 1.5 g acrylamide and 0.03 g methylene bisacryl dissolved in 2.5 ml distilled water.

(ii) 7.9 g 2x recrystallised urea dissolved in 13.2 ml distilled water;

(iii) 0.5 g ammonium persulphate dissolved in 5 ml distilled water.

The gel ingredients consisted of 2 ml of the acrylamide solution (i), 0.8 ml 'Ampholine' (LKB, pH 6-8), 13.2 ml of urea solution (ii), and 10 µl TEMED mixed in a 150 ml vacuum flask under reduced air pressure. 50 µl of ammonium persulphate solution (iii) was added, and the solution was then poured immediately between the two vertically held glass plates. A sample adaptor, (h) in Fig. 2.1, was placed on top of the gel after pouring. Once the gel was set the plastic tape was removed from the bottom of the plates and the gel holder was placed in position in the apparatus.

Sulphuric acid (0.01%) was added to the lower trough to a depth of ca. 4 mm and 0.2% triethanolamine solution was placed between the plates above the sample adaptor. The upper electrode carrier was then lowered into position so that the electrode was parallel with the gel surface.

2.8.3. S-Carboxymethylation of RuBPC-ase

5 mg of RuBPC-ase was suspended in 0.2 ml 0.025 M Tris-HCl buffer (pH 7.4) containing 5 mM EDTA. The 1 cm x 7.5 cm incubation tube and contents were evacuated and the air replaced by N_2, the tube containing the protein crystals then flushed with N_2 for 5 min. Then 0.8 ml of degassed 0.5 M Tris-HCl buffer (pH 8.5) containing 8 M urea and 1 mM EDTA (Tris-urea medium) was introducted by syringe into the sealed tube. As soon as the crystals had dissolved with the aid of gentle shaking, 0.2 ml of Tris-urea medium containing 5 mg of dithiothreitol was injected and incubation allowed to proceed for 2 h at 25°C. The tube was then covered with aluminium foil to exclude light and 0.2 ml Tris-urea medium containing 15 mg iodoacetic acid was injected. After 5-10 min incubation at 25°C, the reaction mixture was passed through a 1 cm x 15 cm Sephadex G25 column previously equilibrated with Tris-urea medium and the protein fraction was collected.

2.8.4. Preparation of the Samples

Two calibration kits were used to determine the pH gradient profile-the Broad Isoelectric Focusing Point (pI) Calibration Kit, pH range 3-10, (Pharmacia) and the High pI Calibration Kit, pH range 5-10.5, which included lentil lectin-basic, lentil lectin-middle, lentil lectin-acidic, myoglobin-basic, myoglobin acidic, and human carbonic anhydrase B as marker molecules. In each case, the content of one vial from the kit was dissolved in 100 µl water; 50 mg twice-recrystallized urea and 1 mg dithiothreitol were added, and the mixture was incubated for 30 min at room temperature. Leaf protein from Medicago falcata was extracted and RuBPC-ase was purified. RuBPC-ase of spinach (Spinacea oleracea) was supplied by Dr. W.

Bottomley (CSIRO). Twice-recrystallized urea and dithiothreitol were added to protein solutions in the amounts indicated above, and incubated for 30 min at room temperature.

2.8.5. Sample Application

Sulphuric acid was added to the lower trough and triethanolamine solution was placed between the plates above the sample adaptor 30 min before the application of samples at 4°C. Sample compartments were loaded with 20 µl solution from the calibration kit together with a calculated volume of RuBPC-ase of spinach and lucerne, by means of a micropipette.

2.8.6. Running Conditions

The apparatus was set to 80 V, 6 mA, with power kept constant below 1.0 watt for the duration of the experiment. The gel was normally pre-run for 15-30 minutes, while the running time for the experiments varied between 4.5 h and 18 h at 4°C.

2.8.7. Gel Staining

Gels were stained for 1-4 h with 0.04% bromophenol blue in a solution containing ethanol, glacial acetic acid, and water in the proportion 10:1:9. Destaining was done with a 6:1:13 mixture of the same solvents.

2.9. TRYPTIC PEPTIDE MAPS

After separation, the LSU and SSU of RuBPC-ase were subjected to trypsin hydrolysis and the tryptic peptides resolved by two-dimensional solvent chromatography and paper electrophoresis by the technique described previously (Kung et al., 1972).

2.10. ISOLATION AND ANALYSIS OF CHLOROPLASTS

2.10.1. Collection of Leaf Discs

Four 2-mm discs were cut from each of the three leaflets, midway between the midrib and leaf margin. The first disc was cut from the lower half of the lamina, the second immediately below it.

2.10.2. Fixation of Leaf Discs

The twelve discs were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.3 and stored at 4°C.

2.10.3. Preparation of Cells for Examination

Six discs of each plant sample were transferred to 3 ml of 5% $K_2Cr_2O_7$ in IN HCl, 60°C, for 2.5 to 3 h, until they could be separated into single cells by gently pipetting up and down in the test tube. This preparation was used to estimate cell number per disc.

The remaining six discs were transferred to 0.05 M EDTA, pH 9.0, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before placing a coverslip on top. The volume should be such that it just fills the area under the coverslip. One-cm brass weights were put on the coverslips to flatten the preparation, and were left for one to two hours. Paper tissue was then folded around the slides to remove any excess moisture and the glass pressed with the heel of the hand or gently tapped with the rounded end of a biro pen to further separate the cells. The slides were finally sealed with clear nail varnish. This preparation was used to determine chloroplast number, plastid diameter and cell area.

2.10.4. Determination of Plastid Number Per Cell

Chloroplast number per cell was determined under a microscope

and the mean of a genotype was based on the average plastid number for 12 cells.

2.10.5. Determination of Plastid Area

The basic measurement for calculation of plastid area was plastid diameter, as measured using an eyepiece micrometer and a Zeiss microscope (× 40 phase plan × 2 optivar, × 10 eyepieces; 16 EPU = 0.2 mm; 1 EPU = 0.02/16 = 1.25 mm).

Calculation of plastid area was as follows :

$$\text{plastid area } (\mu m^2) = \pi/4 \left[1.25 \left(\frac{d_1 + d_2}{2} \right) \right]^2$$

where $\pi = 3.14$ and d_1 and d_2 are the measurements of two diameters of each chloroplast.

For the sake of simplicity the plastid areas were determined with reference to a standard table (Table 2.1 below). This table gives a series of sum values of the two measured diameters ($d_1 + d_2$) and the corresponding plastid areas.

Table 2.1. The sum of diameter units (A) of plastids (d_1+d_2, in μm) and the corresponding plastid areas (B) in μM^2.

A:	2.0	2.5	3.0	3.5	4.0	4.5	5.0
B:	1.227	1.917	2.761	3.758	4.909	6.213	7.670
A:	5.5	6.0	6.5	7.0	7.5	8.0	8.5
B:	9.281	11.045	12.962	15.033	17.275	19.635	22.818
A:	9.0	9.5	10.0	10.5	11.0	11.5	12.0
B:	24.850	27.706	30.680	33.847	37.122	40.601	44.179
A:	12.5	13.0	13.5	14.0	14.5	15.0	15.5
B:	47.969	51.849	55.951	60.132	64.547	69.029	73.757
A:	16.0						
B:	78.540						

2.10.6. Determination of Cell Number Per Disc

The number of cells in a volume of 3.2 μl on a haemocytometer slide was counted, six counts per sample, and the number of cells per 2 mm diameter disc calculated.

2.10.7. Determination of Cell Area

The cell preparations were photographed and the film printed as described in Chapter 2.14. The circumference of each cell was measured by a planimeter wheel attached to a programmed computer (Hewlett Packard 9845) which estimated the area of each cell. The cell area of a genotype was taken on the mean of 24 measurements.

2.11. TEMPERATURE AND LIGHT TREATMENTS

Seeds of HP and MF genotypes were germinated in petri dishes in the presence of a Rhizobium spp. bacterial culture (Nodulaid inoculant, Group "A", from Agricultural Laboratories, Carlingford Road, Sefton, N.S.W.). After germination (3-4 days), they were transplanted at about 1 cm depth into 15 cm pots filled with perlite. Twelve pots of each genotype were placed in phytotron cabinets at 35/30°C and 25/19°C and the plants were grown under normal daylight conditions until harvested. The plants were close to green bud stage when harvested.

Plants of the two genotypes grown at 25/19°C under normal day light conditions were transferred to a low-daylight cabinet (12 $\mu E.m^{-2}sec^{-1}$) with 12 h day length at 25/19°C and were kept in the cabinet for one to four days.

2.12. SCANNING OF STAINED GELS

The stained gels were cut into 1-cm wide slices along the vertical length of the gel and scanned in a Gilford spectrophotometer 240. The wave length was set at 600 nm and the absorbance control was 1500.

2.13. ELECTRON MICROSCOPY

Leaf segments were chopped into fixative (2.5% glutaraldehyde in 35 mM Na PIPES buffer, pH 8, 5 mM $MgCl_2$) and left overnight at 4°C. Fixed tissue was washed in PIPES buffer (50 mM, pH 6.8), post-fixed in $0s0_4$ (1:1 mixture of 5% aq. $0s0_4$ solution and 50 mM Na PIPES buffer, pH 6.5), washed in dist. H_20, and dehydrated using an ethanol concentration series. Infiltration and embedding in Spurr's Resin A (firm) (Spurr 1969) were done using propylene oxide. Sections were cut on a Reichert 0MU3 ultramicrotome,

grid-stained using 2% barium permanganate (Pyliotis, 1974), and examined in a JEOL 100C electron microscope.

2.14. STATISTICS

The standard analysis of variance was used in the majority of caculations.

The realized heritability was determined as follows

$$h = \frac{R}{S}$$

where h^2 = realised heritability, R = response to selection and S = selection differential.

The selection response was calculated from the means of the protein content of two consecutive generations (Falconer, 1964).

2.15. PHOTOGRAPHY

A polaroid camera was used for most of the gel photography. The type of film was 665 positive/negative Land Pack Film. Other technical details were : exposure time between 1/8 and 1/15 sec; lens aperture between 5.6 and 8 "f" setting; development time of the positive between 30 to 45 sec. The developed negatives were placed in Na_2S0_3 solution (136 g l^{-1}) for two hours, followed by rinsing in running cold water overnight. After drying the negatives, prints were made.

A Zeiss microscope (see section 2.10 for details) and Kodak Panatomic-X film were used for photography of cells and chloroplasts. The negatives were developed, fixed, and dried using standard methods. Ilfospeed No. 3 paper was used for prints. The developing solution contained water and Ilford Ilfospeed developer at

a 1:9 ratio. Development time was 1 min. The fixative was Ilford Hypan Rapid Fixer (1:4 ratio of water to fixative); prints were kept in fixative for 3 min., rinsed in tap water for 15 min, and dried overnight.

All the other photography (black-white and colour) was done by standard photographic techniques.

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CHAPTER 3.

PRELIMINARY CHROMATOGRAPHIC ISOLATION AND CHARACTERIZATION OF RuPBC-ase IN MEDICAGO

3.1. INTRODUCTION

A physico-chemical examination of RuBPC-ase from M. sativa was carried out by Noguchi et al. (1978). They isolated RuBPC-ase from leaves by Sephadex G-25 column chromatography followed by polyacrylamide gel electrophoresis. Analytical ultracentrifugation gave a $S^{\circ}_{20,w}$ value of 18.0, which was close to 536 kd. X-ray diffraction analysis indicated that the molecules of RuBPC-ase are roughly 115 x 115 x 100 Å in size, with a stoichiometry of eight LSU and eight SSU polypeptides. According to electron micrographs the RuBPC-ase molecule is square-shaped with a hole in the middle and the SSU polypeptides are located at the outer periphery of the LSU polypeptides. SDS-gel electrophoresis revealed two sizes of subunits. Molecular weights were estimated at 53 kd and 14 kd for LSU and SSU polypeptides respectively.

Macromolecular properties and subunit interactions of RuBPC-ase from M. sativa were examined by Tomimatsu (1980). Enzyme activity, light-scattering circular dichroism, and differential scanning colorimetric characters were studied. The enzyme was quite stable, showing no loss in activity after a year's storage, either at 4°C in buffer (0.02 M potassium phosphate, pH 7.5) solution or at -20°C as a precipitate in 60% $(NH_4)_2SO_4$. Lyophilization of a salt-free solution of the enzyme produced mostly an insoluble product; lyophilization of a buffer solution of the enzyme produced a soluble product which retained most of its activity. The molecular weight as determined by light scattering is 497 kd and in buffer the enzyme neither

dissociated into subunits at low concentration (6 μg/ml), nor formed molecular aggregates at high concentration (15 $\mu g/ml^{-1}$). Treatment with p-chloromercuribenzoate at pH 7.5 and subsequent storage at pH 9 did not dissociate the subunit structure. In solution at pH 7.5, the enzyme had a compact conformation and helices made up 29% of the secondary structure. At pH 3.4, it was largely unfolded, but the subunits were still associated, and ß-structure and disordered structures dominated the intrinsic region CD spectrum. The enzyme denatured at 76.2°C and had an enthalpy of denaturation of 6.3 cal g^{-1}. The presence of a single endotherm suggests, that the subunits are tightly bound to each other and are denatured as single units.

The synthesis of RuBPC-ase is reported to be regulated by both nuclear and chloroplast genes in such a way that the synthesis of SSU polypeptide is under nuclear control and that of LSU polypeptide is under chloroplast DNA control (Kung, 1976). The polypeptide composition of RuBPC-ase of several species of plants displayed a considerable array of variation. Chen et al. (1977) reported that M. sativa had three LSU polypeptides and one acid SSU polypeptide.

The aims of Chapter 3 are as follows: firstly, to describe and characterize the total leaf protein of HR genotype; secondly, to determine the polypeptide composition of RuBPC-ase of HR, HP and MF genotypes; thirdly to compare the tryptic peptide maps of LSU and SSU polypeptides of HR and MF genotypes.

3.2. RESULTS

3.2.1. Isolation and Analysis of Leaf Protein

3.2.1. (i) Partitioning of Leaf Proteins by Sepharose 6B Chromatography

Sepharose 6B column chromatography of soluble lucerne leaf protein gave an elution profile with four distinct protein peaks for the HR cultivar (Fig. 3.1.). The first peak was eluted shortly after the void volume (Dextran Blue peak). This fraction had a molecular weight of 2×10^6 dalton and had a higher absorbance at 260 nm than at 280 nm. The second peak (fractions 36 to 43) had a 280/260 nm ratio of 1.7 and a molecular weight of 530 kd. The third peak (fractions 43 to 54) had a 280/260 nm ratio of 1.4. The fourth peak (between fractions 54 and 70) appeared to contain most of the phenolic substances in the sample and was yellow in colour; its absorbance at 260 nm was similar to that at 280 nm.

Proteins from the four peaks eluted from the Sepharose 6B column were dialysed against water overnight and freeze dried in order to determine dry matter yields and protein (Kjeldahl nitrogen) contents (Table 3.1). The second peak contained the largest amount of protein (7.99 mg protein/5 ml sample solution) while the third had approximately half that amount (4.00 mg protein/5 ml sample solution). The recovered protein represented 2.43% of plant dry matter and 22.5% of the total protein of the HR genotype. The second peak represented 57% of the total extracted leaf protein.

Samples of the freeze-dried products of the second, third and fourth peaks are shown in Fig. 3.2. The colours of those products are white (a,b) and tan (c), respectively.

Fig. 3.1. Sepharose 6B chromatography elution profile of lucerne leaf proteins from HR genotype plants.

A Sepharose 6B column (1.5 x 86 cm) was used in this experiment. Fractionation was carried out at 4°C using 20 mM Tris-HCl buffer, pH 6.8, containing 50 mM NaCl and 0.02% sodium azide. The flow rate was 0.1 ml min^{-1} and each eluted fraction was 2.5 ml. 0.1 gm of dextran blue was dissolved in 3 ml of the above buffer and applied to the Sepharose 6B column to determine the void volume. Five ml of supernatant of leaf extract (see preparation, Chapter 2.4.2.) was applied to the column, and the eluted fractions were collected between tubes 20-70, using a Gilson fraction collector (TDC 80).

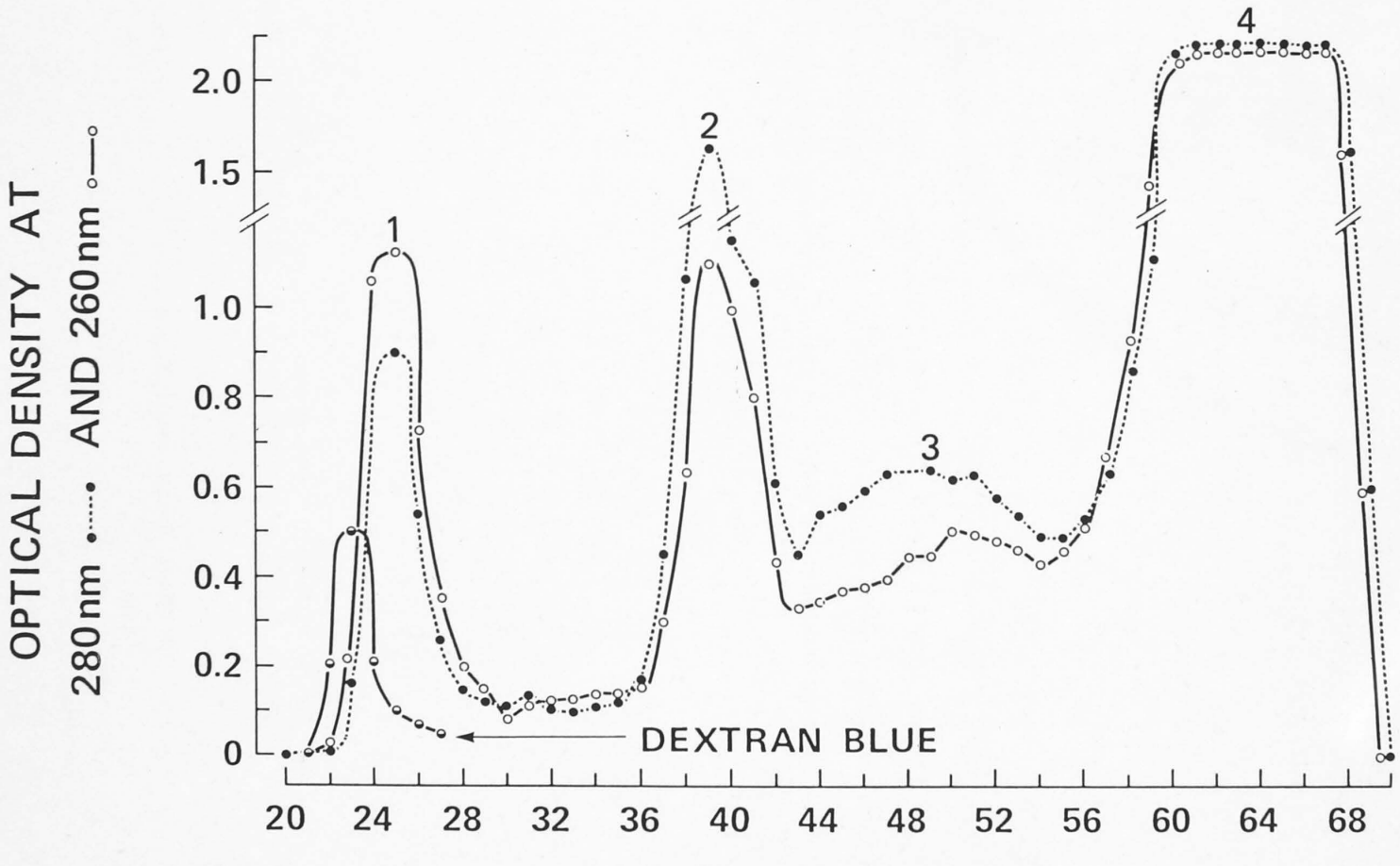

OPTICAL DENSITY AT
280nm •---• AND 260nm ○—○
2.0
1.5
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
DEXTRAN BLUE
20
24
28
32
36
40
44
48
52
56
60
64
68
FRACTION NUMBER

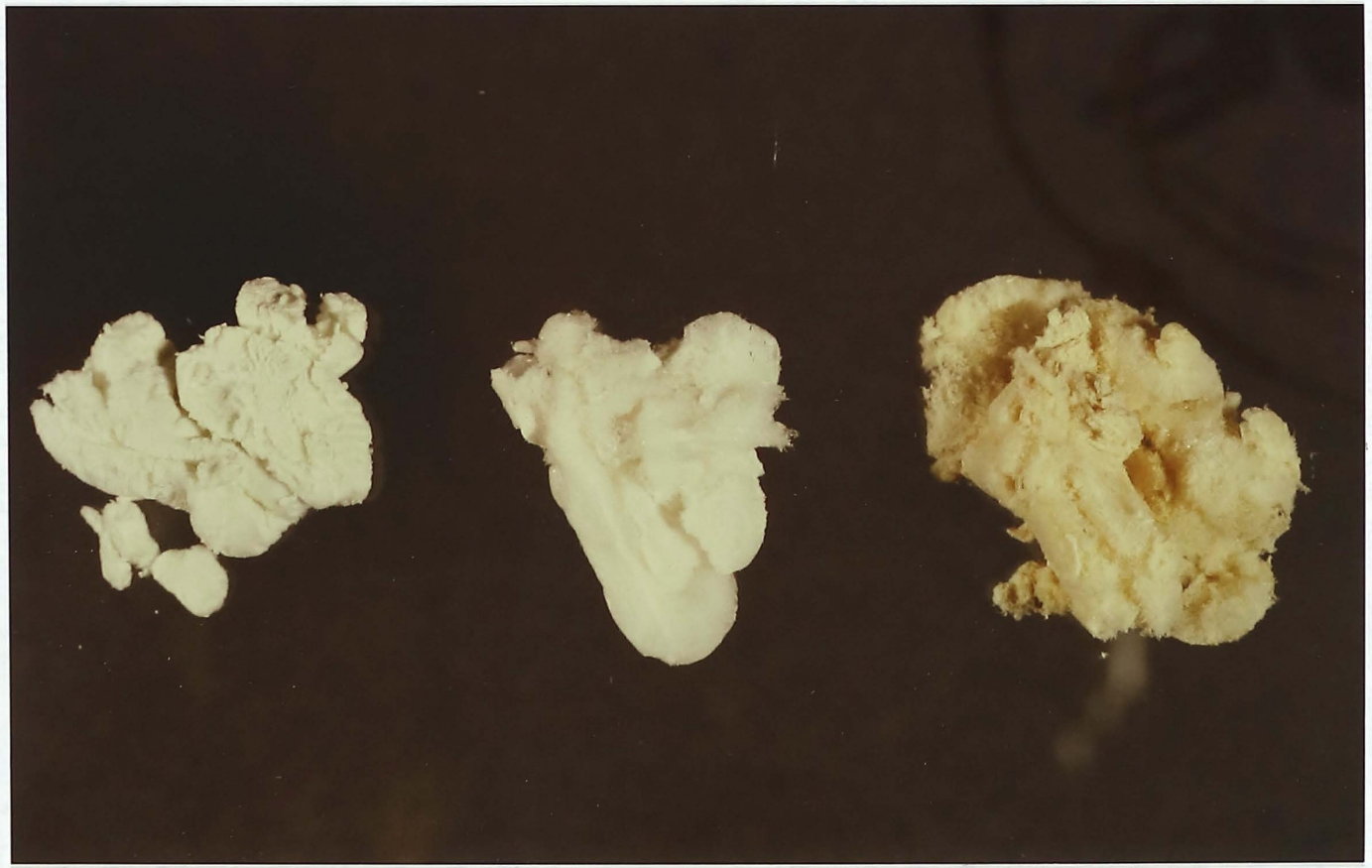
Table 3.1. Dry matter and protein content of four peaks of leaf proteins from a Sepharose 6B chromatographic column. Proteins from the four peaks eluted from the Sepharose 6B column (Chapter 2.4.2) were dialysed against water overnight and freeze-dried, to determine dry matter yields and nitrogen content (Chapter 2.7).

Table 3.1. Dry matter and protein content of the four peaks of leaf proteins from a Sepharose 6B chromatographic column.

Peaks	Dry Matter (mg)	Protein (mg)
First	0.5	0.19
Second	16.2	7.99
Third	15.0	4.00
Fourth	19.8	1.85

Fig. 3.2. Freeze dried extracts of the second (a), third (b), and fourth (c) peaks of leaf proteins of HR genotype plants from Sepharose 6B column chromatography.

5 ml of supernatant of leaf extract of HR genotype (see Chapter 2.4.2.) was applied to the column and the second, third and fourth elution peaks were collected. They were dialysed against water overnight and freeze dried.



a b c

3.2.1.(ii) SDS Gel Electrophoresis of Leaf Protein Fractions

The polypeptide composition of the four peaks differed markedly on SDS polyacrylamide gels (Fig. 3.3). Two weak bands were found in material from the first peak (lane 1). Fractions 38 and 40 from the second peak each showed the same two bands as well as several minor bands (lanes 2,3) and fraction 42 from the same peak had additional minor bands (lane 4). The band at 53 kd is LSU and that at 14 kd is SSU of RuBPC-ase (Noguchi 1978). In contrast, the fractions from the third peak were similar, showing many bands distributed along the gel (the banding patterns of fractions 47 and 49 are given in (lanes 5 and 6, respectively). The fourth peak also contained a number of major and minor polypeptides, the majority of which appeared in the lower molecular weight (< 15 kd) range (lane 7).

3.2.1. (iii) Enzyme Assay of Chloroplast and "Cytoplasmic"* Proteins

The results of SDS polyacrylamide gel electrophoresis suggest that the second peak of the Sepharose 6B profile consists largely of RuBPC-ase while the third peak contains many smaller leaf proteins (multiple bands). This conclusion is supported by the results of assays for RuBPC-ase activity. The two samples of the second peak protein showed high levels of RuBPC-ase activity (0.25 and 0.1 μmol $C0_2$ $min^{-1}mg^{-1}$ protein, respectively). There was no carboxylase activity in peak 3. The proteins (50 μg each) of peaks 2 and 3 were also fractionated by starch gel electrophoresis and then assayed for a range of enzyme activities (Fig. 3.4 and Table 3.2). Peak 2 showed no detectable activity for any of the other enzymes surveyed, but all eleven enzymes occurred among the proteins of peak 3.

*"cytoplasmic" proteins also included some small MW chloroplast proteins.

Fig. 3.3. SDS gel electrophoretograms of HR genotype plant leaf extract fractions from four chromatography column peaks : lane 1,
first peak; lanes 2, 3, 4 are fractions 38, 40, 42 of the second peak; lanes 5, 6 are fractions 47, 49 of the third peak; lane 7, fourth peak.

Protein of the four fractions from a Sepharose 6B column was precipitated with 10% TCA and then carboxymethylated. The pellets were dissolved in 70 µl of a solution containing 0.15 g SDS, 0.8 g sucrose, 100 µl 2-mercaptoethanol and 0.01 g bromophenol-blue per 10 ml of Tris-glycine buffer, pH 8.2, by heating in boiling water for two minutes.

Proteins were separated on 10% acrylamide SDS gels (Thomson and Schroeder, 1978) using a Gradipore electrophoresis apparatus. The gel buffer was 25 mM Tris (pH 8.2), 200 mM glycine, while the upper tray contained the same buffer with 0.1% SDS. The gels were pre-run for 2 h at approx. 150 V, 30 mA prior to sample application. These were then run at approx. 150 V and 30 mA constant current for 1.5 h. Proteins were stained with Coomassie blue solution. Protein molecular size for bands on the electrophoretogram is calibrated in kilodaltons (kd). LSU = 53 kd, SSU = 14 kd.

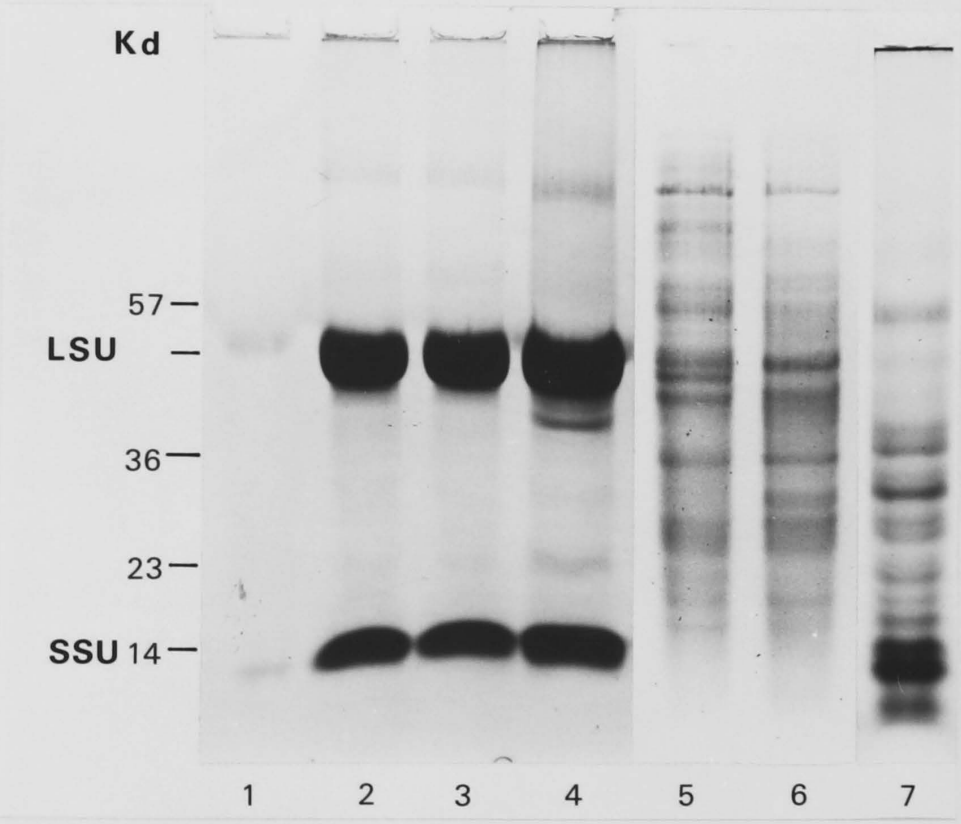
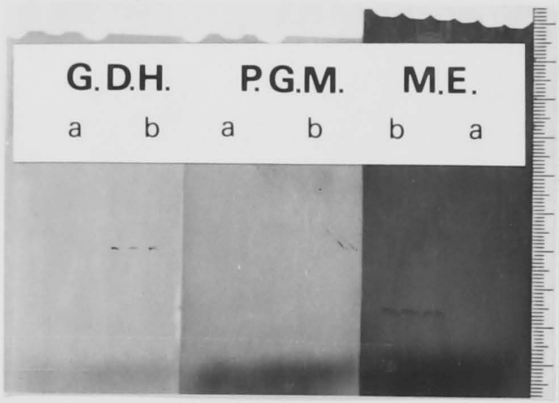
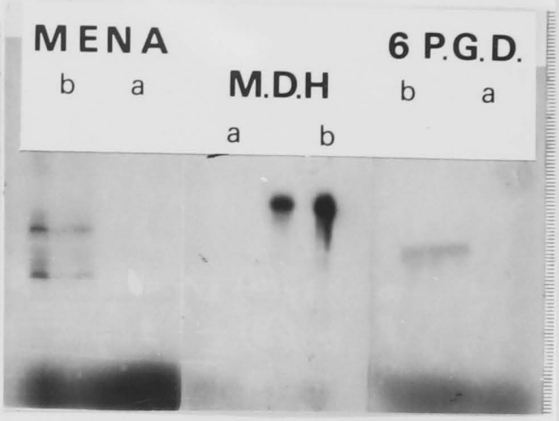

Kd
57
LSU
36
23
SSU 14
1
2
3
4
5
6
7

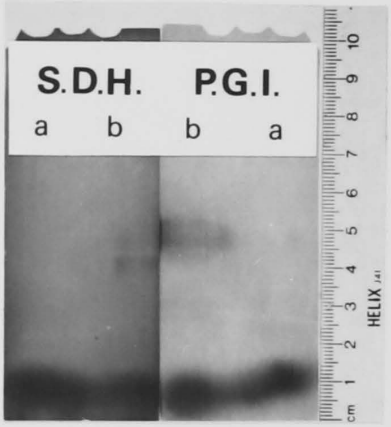
Fig. 3.4. Enzyme activity of peak two (a) and peak three (b) proteins from a Sepharose 6B column (see Fig. 3.1). 50 µl of peak two and peak three proteins were assayed by starch gel electrophoresis.

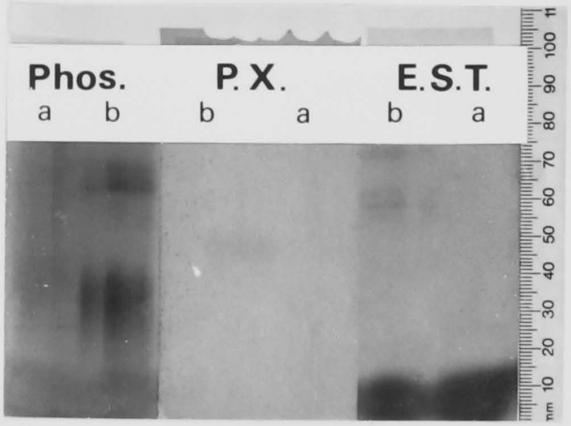
Electrophoresis was conducted in a discontinuous buffer system (pH 8.0) using 13% starch (Shaw and Koen, 1968). Running conditions were those described previously (Marshall and Allard, 1970). At the completion of electrophoresis, each gel was cut vertically into three slices and each slice was stained for a different enzyme (Brewer and Sing, 1970 and Brown et al. 1978). (See Table 3.2 for list of enzymes).


G.D.H.
a b
P.G.M.
a b
M.E.
b a


MENA
b a
M.D.H
a b
6 P.G.D.
b a


I.P.O.
a b
L.A.P.
a b
G.O.T.
a b


S.D.H.
a b
P.G.I.
b a


Phos.
a b
P.X.
b a
E.S.T.
b a

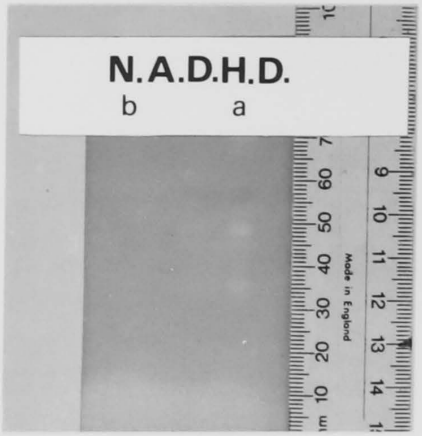

N.A.D.H.D.
b a

Table 3.2. Enzyme assay (activity) of peak 2 and peak 3 proteins derived from Sepharose 6B chromatography of lucerne extracts.

Samples of protein from the second and third peaks eluted from Sepharose 6B columns were electrophoresed on starch gel and stained for a range of enzymes. Electrophoresis was conducted in a discontinuous buffer system (pH 8.0) using 13% starch gels (Shaw and Koen 1968). Running conditions were those described previously (Marshall and Allard 1970). At the completion of electrophoresis, each gel was cut horizontally into three slices and each slice was stained for a different enzyme. The staining procedures were similar to those described by Brewer and Sing (1970) and Brown et al. (1978).

Table 3.2. Enzyme Assay (activity) of Peak 2 and Peak 3 Proteins Derived from Sepharose 6B Chromatography of Lucerne Leaf Extracts.

Enzyme	Peak 2	Peak 3
Malate dehydrogenase (NAD^+) (MDH)	no bands	one band
6 Phosphogluconate dehydrogenase (6PGD)	no bands	one band
Shikimate dehydrogenase (SDH)	no bands	two bands
Glucophosphate isomerase (PGI)	no bands	one band
Glutamate dehydrogenase (GDH)	no bands	one band
Acid phosphatase (ACPH) (PHOS)	no bands	two bands
Indophenol oxidase (IP0)	no bands	one band
Peroxidase (PER) (PX)	no bands	two bands
Esterase (EST)	no bands	two bands
Glutamate oxaloacetate transaminase (GOT)	no bands	two bands
NADH diaphorase (NADHD)	no bands	two bands
Phosphoglucomutase (PGM)	no bands	one band
Malic enzyme, malate dehydrogenase ($NADP^+$) (ME)	no bands	one band
Leucine aminopeptidase (LAP)	no bands	two bands
Menadione reductase NADH dehydrogenase (MENA)	no bands	four bands

3.2.1. (iv) Amino Acid Analysis of Chloroplast and "Cytoplasmic" Proteins

The amino acid composition of the chloroplast (second peak) and "cytoplasmic" (third peak) proteins is given in Table 3.3. The FAO provisional recommendations for the minimum levels of the essential amino acids are also included in this table for comparative purposes. These results show that with the exception of methionine (slightly below the recommended level) all the essential amino acids are adequately represented in both proteins. The chloroplast fraction contains marginally greater amounts of methionine than the "cytoplasmic" fraction.

3.2.1. (v) Further Purification of RuBPC-ase by DE-52 Column Chromatography

The protein of the second peak from Sepharose 6B separation was further purified using DE-52 column chromatography with a 0.1 - 0.5 M NaCl gradient. The major proteins were eluted between fractions 20-40 with a NaCl concentration of 0.12-0.25 (Fig. 3.5). Although there is a single major elution peak (between fractions 20-30) it appears that there is also a shoulder peak eluted between fractions 32 and 38. In addition, minor contaminant protein peaks were found between fractions 5 and 20 and between 40 and 50.

SDS gel electrophoresis of DE-52 purified proteins from fractions 20-40 indicated that they consisted of four major subunits (three with a molecular weight of approximately 53 kd daltons and one with molecular weight of about 14 kd) as well as some minor bands (Fig. 3.6).

Table 3.3. Percent amino acid composition of fractionated chloroplast and "cytoplasmic" leaf protein of HR genotype.

Chloroplast and "cytoplasmic" leaf proteins were isolated by Sepharose 6B column chromatography (Chapter 2.4.2). Amino acid compositions were determined by the methods of Byers (1971).

Table 3.3. Percent amino acid composition of fractionated chloroplast and "cytoplasmic" leaf protein of HR genotype.

Amino Acids	Chloroplast fraction	"Cytoplasmic" fraction	F.A.O. provisional recommendation of essential amino acids
Lysine	6.82	7.73	4.2
Histidine	3.13	2.35	
Arginine	8.54	5.03	
Aspartic Acid	9.60	12.00	
Threonine	6.01	5.24	2.8
Serine	3.53	5.45	
Glutamic Acid	11.96	13.11	
Proline	4.04	3.92	
Glycine	5.49	5.15	
Alanine	6.43	6.06	
Half Cystine	1.73	1.88	
Valine	5.72	6.25	4.2
Methionine	2.07	1.89	2.2
Isoleucine	4.10	5.26	4.2
Leucine	9.15	9.32	4.8
Tyrosine	5.40	4.17	2.8
Phenylalanine	6.19	5.09	2.8

Fig. 3.5. Diagram of elution profile of RuBPC-ase of HR plant genotype from DE-52 column chromatography. The DEAE-cellulose (DE-52) column (1.5 x 20 cm) was equilibrated with 50 mM Tris-HCl buffer, and eluted with a gradient of 0.1 to 0.5 M NaCl in the same buffer at 4°C. The flow rate was 0.1 ml per min. One ml fractions were collected and monitored for protein content by measuring absorbance at 280 nm.

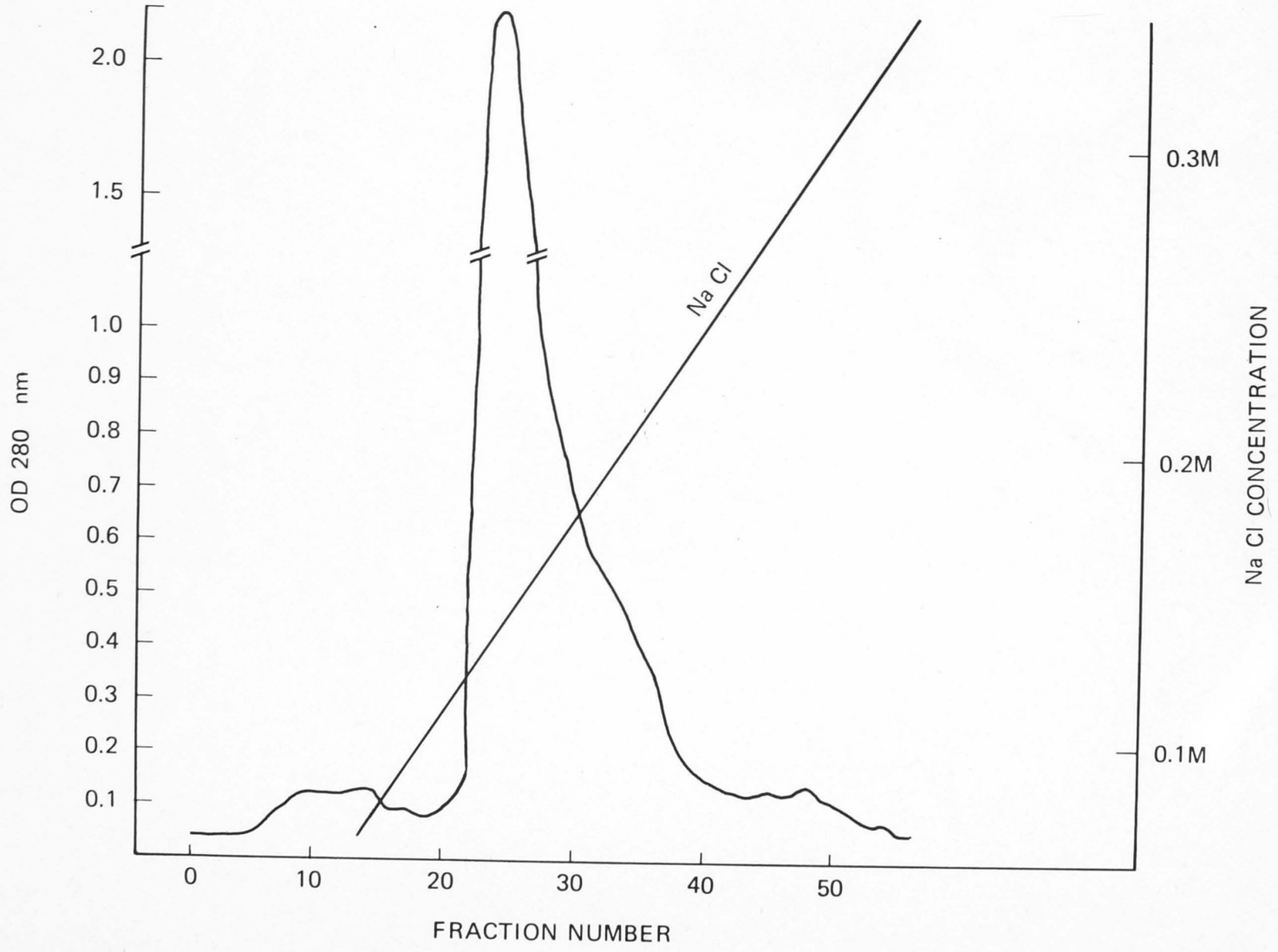
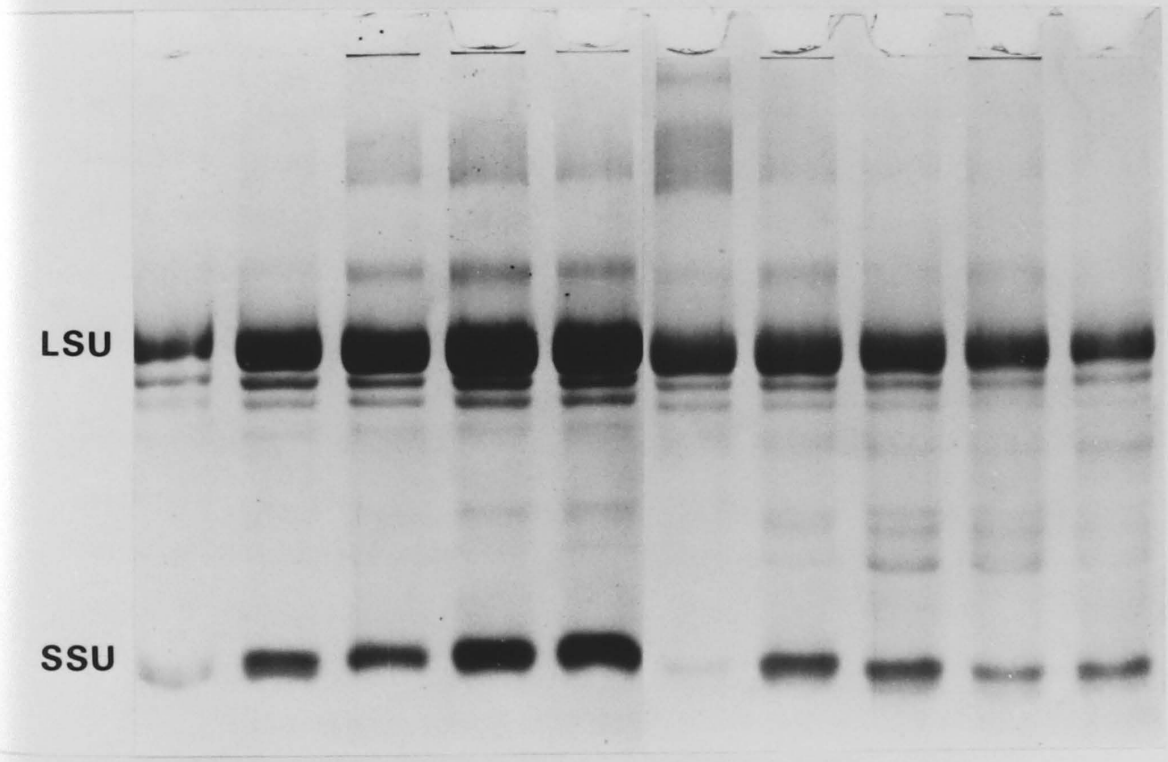

2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OD 280 nm
Na Cl
0.3M
0.2M
0.1M
Na Cl CONCENTRATION
0
10
20
30
40
50
FRACTION NUMBER

Fig. 3.6. SDS gel electrophoetograms pattern of RuBPC-ase from DE-52 column chromatography.

The protein fractions (fractions 20-30, left to right gel slots) from the main electron peak of the DE-52 column were precipitated with 10% (w/v) trichloracetic acid (TCA) at 4°C for 2 hours and centrifuged at 12,000 g for 10 min. The supernatant was discarded and the pellet washed twice with cold 10% TCA. The pellet was dissolved in 70 µl of a solution containing 0.15 g SDS, 0.8 g sucrose, 100 µl mercaptoethanol and 0.01 g bromophenol-blue per 10 ml of Tris-glycine buffer, pH 8.2, by heating in boiling water for 2 min.

Proteins were separated on a 10% acrylamide SDS gel (Thomson and Schroeder, 1978) using a Gradipore electrophoresis apparatus (Scientific and Research Equipment, Sydney). The gel buffer was 25 mM Tris (pH 8.2), 200 mM glycine, while the upper tray contained the same buffer with 0.1% SDS. The gels were pre-run for 2 h at approx. 150 V, 30 mA prior to sample application. The gels were then run at approx. 150 V and 30 mA constant current for 1.5 h. Proteins were stained with Coomassie blue solution. Electrophoretogram shows LSU and SSU polypeptides.


LSU
SSU

3.2.2. Isoelectric Focusing

3.2.2. (i) Effect of Running Time on Isoelectric Focusing of Marker Molecules

The Broad Range pI Calibration Kit was used to test the effect of running time on the pI profile. The marker molecules were as follows: lentil lectin-middle, lentil lectin-acid, myoglobin-basic, myoglobin-acidic, human carbonic anhydrase B, and bovine carbonic anhydrase B. All the marker molecules were separated well on the isoelectric focusing gel, but the three running times 4.5 h, 9 h and 18 h resulted in quite distinct differences with 9 h running time giving the most clear-cut and precise bands (Fig. 3.7, lane 1). After 4.5 h polypeptides were not well focused, while after 18 h some of the markers stained only very faintly. The effect of electrofocusing time on the migration of marker molecules is shown graphically in Fig. 3.8. The migration distances of the bovine carbonic anhydrase B molecules were 2.3, 2.7 and 4.6 cm after 4.5, 9 and 18 hours isoelectric focusing.

3.2.2. (ii) Isoelectric Focusing of RuBPC-ase of Lucerne and Spinach

The calibration experiment using the High pI Calibration Kit also included samples of purified S-carboxymethylated RuBPC-ase from spinach and from lucerne. Four loadings of spinach enzyme (14, 35, 50 and 70 µg) and one (60 µg) of lucerne enzyme were tested. The resultant IEF patterns are shown in Fig. 3.9, lanes 2,3. Both spinach and lucerne of RuBPC-ase have 3 clear bands, representing the LSU of the enzyme from both species which migrated to exactly the same positions on the gel. The SSU showed quite different migration patterns. In the case of lucerne the SSU's did not migrate

Fig. 3.7. Isoelectric focusing separation of the marker molecules of the Broad Range (pH 5.0-10.3) Calibration Kit and LKB Ampholine (pH 6-8) after runs of 4.5, 9 and 18 h (top to bottom).

The content of one vial from the kit was dissolved in 100 µl water; 50 mg twice-recrytallized urea and 1 mg dithiothreitol were added, and the sample then incubated for 30 min at room temperature. The marker mixture sample was applied to gel slot 1, for comparison with spinach RuBPC-ase (slot 2) and lucerne RuBPC-ase (slot 3), each set of 3 slots being replicated 4 times as seen in the figure.

Sulphuric acid was added to the lower trough and triethanolamine solution was placed between the plates above the sample adaptor 30 min before the application of samples at 4°C. Sample compartments were loaded with 20 µl solution from the calibration kit by means of a micropipette.

The apparatus was set initially to 80 V and 6 mA, with power being kept constant below 1.0 W for the duration of the experiment. The gel was normally pre-run for 15-30 minutes, while the running time for the experiments varied between 4.5 h and 18 h at 4°C.

Gels were stained for 1-4 h with 0.04% bromophenol blue in a solution containing ethanol, glacial acetic acid, and water in the proportion 10:1:9. Destaining was done with a 6:1:13 mixture of the same solvents.

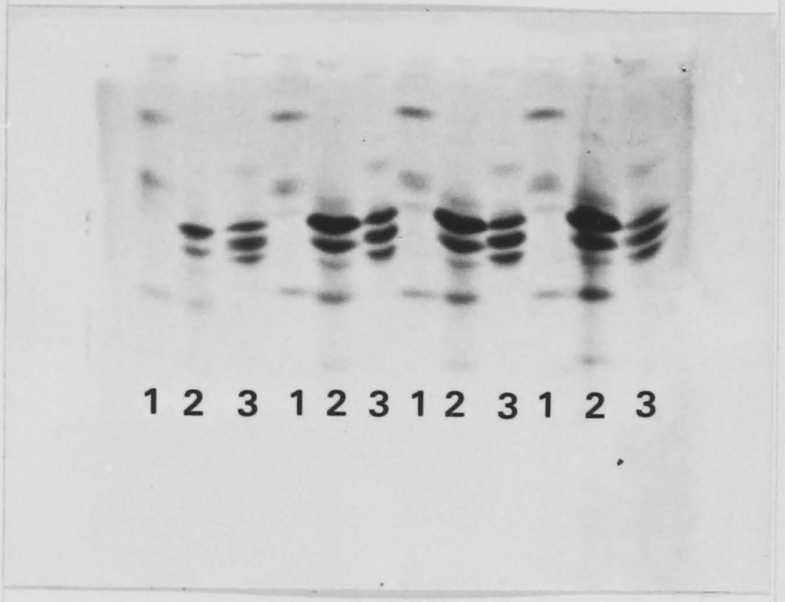

1 2 3 1 2 3 1 2 3 1 2 3

Fig. 3.8. Effect of running time on the migration of marker molecules using a Broad Range (pH 3-10) pI Calibration Kit and LKB ampholine (pH 6-8).

Experimental details were as described for Fig. 3.7, and the distances of migration from the cathode were plotted against the pH gradient.

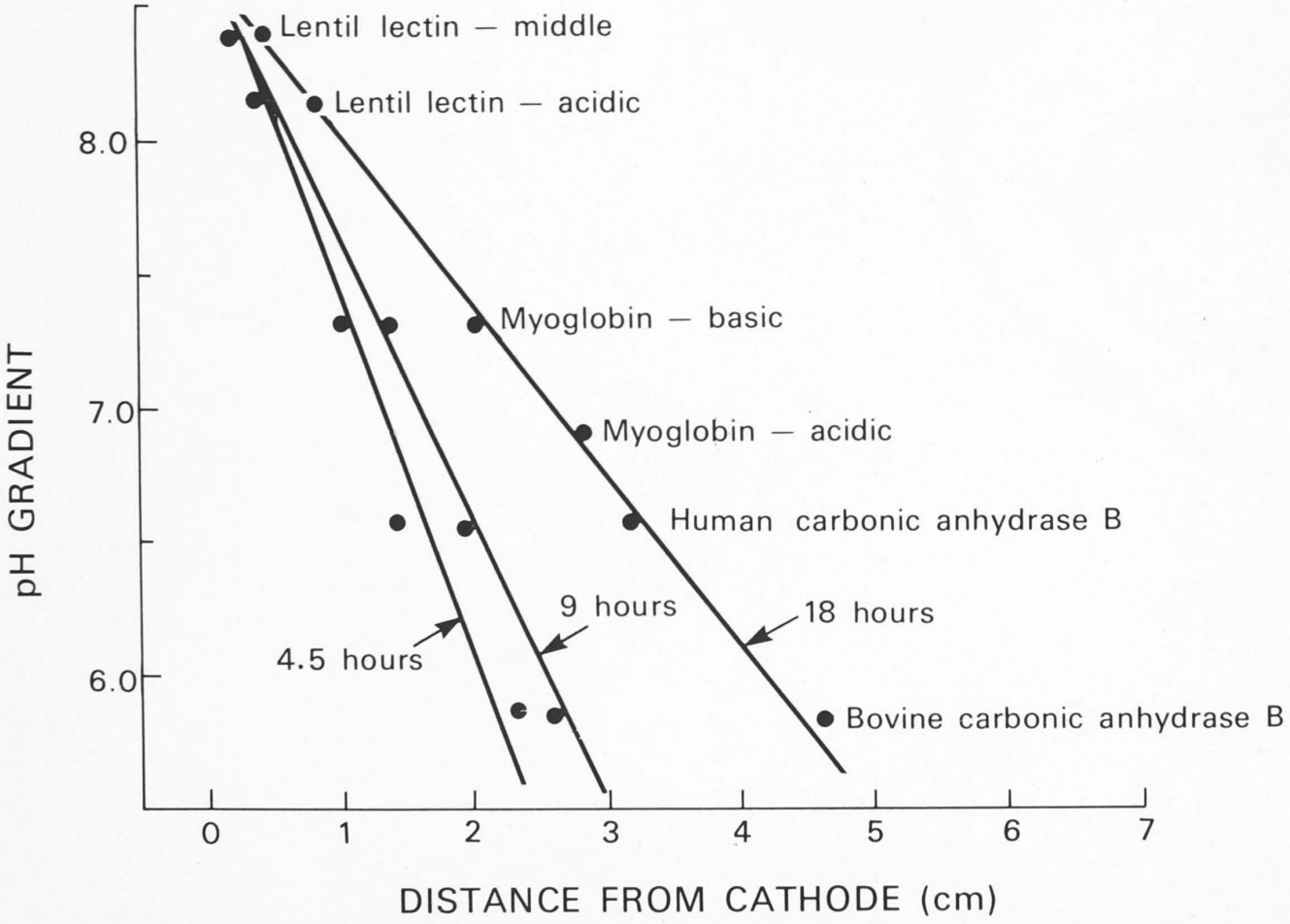
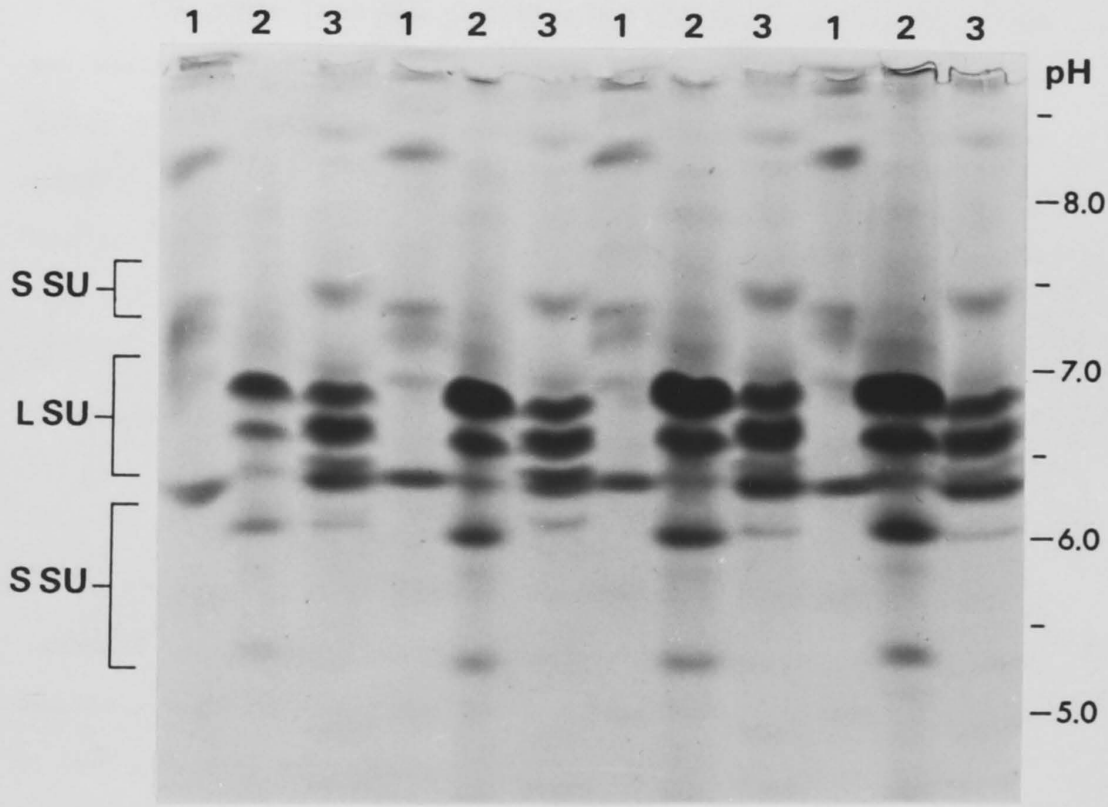

Lentil lectin — middle
Lentil lectin — acidic
Myoglobin — basic
Myoglobin — acidic
Human carbonic anhydrase B
Bovine carbonic anhydrase B
9 hours
18 hours
4.5 hours
8.0
7.0
6.0
pH GRADIENT
0
1
2
3
4
5
6
7
DISTANCE FROM CATHODE (cm)

Fig. 3.9. Isoelectric focusing separation of marker molecules of a High Range (pH 5.0-10.3) pI Calibration Kit (1), RuBPC-ase of spinach (2), and RuBPC-ase of lucerne (3). Sample sizes : marker molecules 5 mg; spinach, 14, 35, 50 and 70 µg; lucerne 60 µg RuBPC-ase.

All samples were S-carboxymethylated. Duration of the experiment was 9 h.

Details of methods are described under Fig. 3.7.


1 2 3 1 2 3 1 2 3 1 2 3
pH
8.0
7.0
6.0
5.0
S SU
L SU
S SU

as far as the LSU towards the anode, while the spinach SSU's migrated further than the LSU.

The calibration pH profile for the High pI Calibration Kit, running for 9 h under the standard conditions, and the positions of the subunits of RuBPC-ase of spinach and lucerne are shown in Fig. 3.10. It is clear from the plot that the SSU's of spinach lie on the extrapolated (dashed) portion of the calibration curve. Isoelectric points of the RuBPC-ase subunits were estimated from the migration pattern on the gel. The results strongly suggest equivalence of the LSU pI values in both species and divergence of the SSU pI values. The LSU bands lie between pI 6.6 and 6.9 for both species. The SSU for spinach has 2 bands at 6.15 and 5.45, and the SSU for lucerne has one band at pI 7.45.

3.2.2. (iii) Separation of Subunits of RuBPC-ase by Sephadex G100 Chromatography

RuBPC-ase of HR genotype plants was S-carboxymethylated, dissolved in Tris-HCl buffer containing 0.5% SDS and applied to a Sephadex G100 column. Fig. 3.11. shows the quantitative separation of LSU and SSU as measured by optical density at 280 nm. The LSU was eluted between fractions 20-30 and the SSU was found between fractions 34-44. The ratio of LSU to SSU was very close to 4:1.

LSU and SSU polypeptides of RuBPC-ase of HR and MF genotypes were separated by G-100 column chromatography. The results of SDS gel electrophoresis of RuBPC-ase, LSU and SSU polypeptides are presented in Fig. 3.12. Lane 1 shows the LSU and SSU bands of RuBPC-ase of HR genotype. The LSU polypeptide (lane 2) has at least six bands between the origin of the gel and the smaller molecular band corresponding to the LSU polypeptide (53 kd)

Fig. 3.10. Comparison of the pI of subunits of RuBPC-ase of spinach (Sp) and lucerne (Lu) with markers of the High Range (pH 5-10.3) Calibration Kit.

The data from the isoelectric focusing gel of Fig. 3.9 were utilized and the migration distances from the cathode were plotted against the pH gradient.

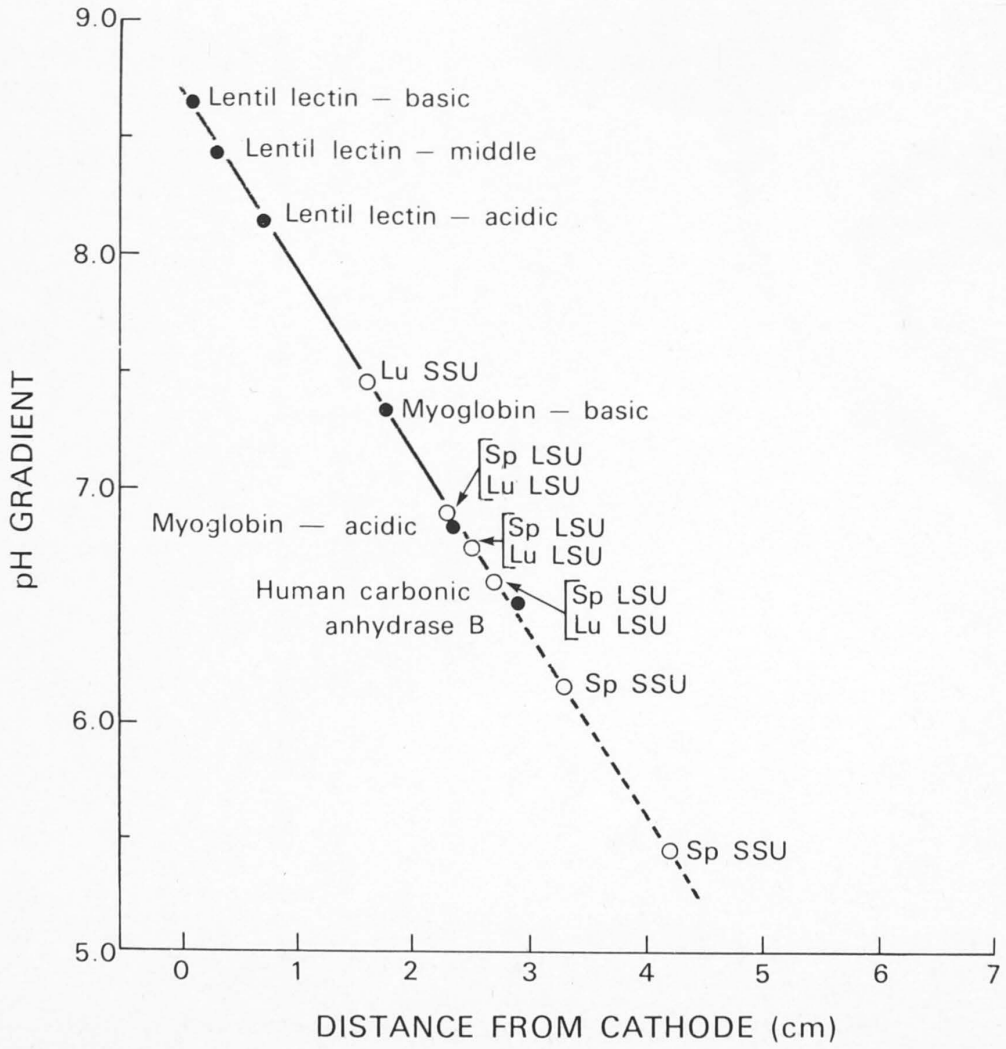

9.0
8.0
7.0
6.0
5.0
pH GRADIENT
Lentil lectin — basic
Lentil lectin — middle
Lentil lectin — acidic
Lu SSU
Myoglobin — basic
Sp LSU
Lu LSU
Myoglobin — acidic
Sp LSU
Lu LSU
Human carbonic
anhydrase B
Sp LSU
Lu LSU
Sp SSU
Sp SSU
0
1
2
3
4
5
6
7
DISTANCE FROM CATHODE (cm)

Fig. 3.11. Elution profile of LSU and SSU of HR genotype plants from Sephadex G100 column chromatography.

Precipitated and carboxymethylated RuBPC-ase was dissolved in 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 percent sodium dodecylsulfate and was passed through a 2.5 cm x 85 cm Sephadex G100 column previously equilibrated with the same buffer. The flow rate was 0.1 ml/min and the volume of each fraction was 1 ml.

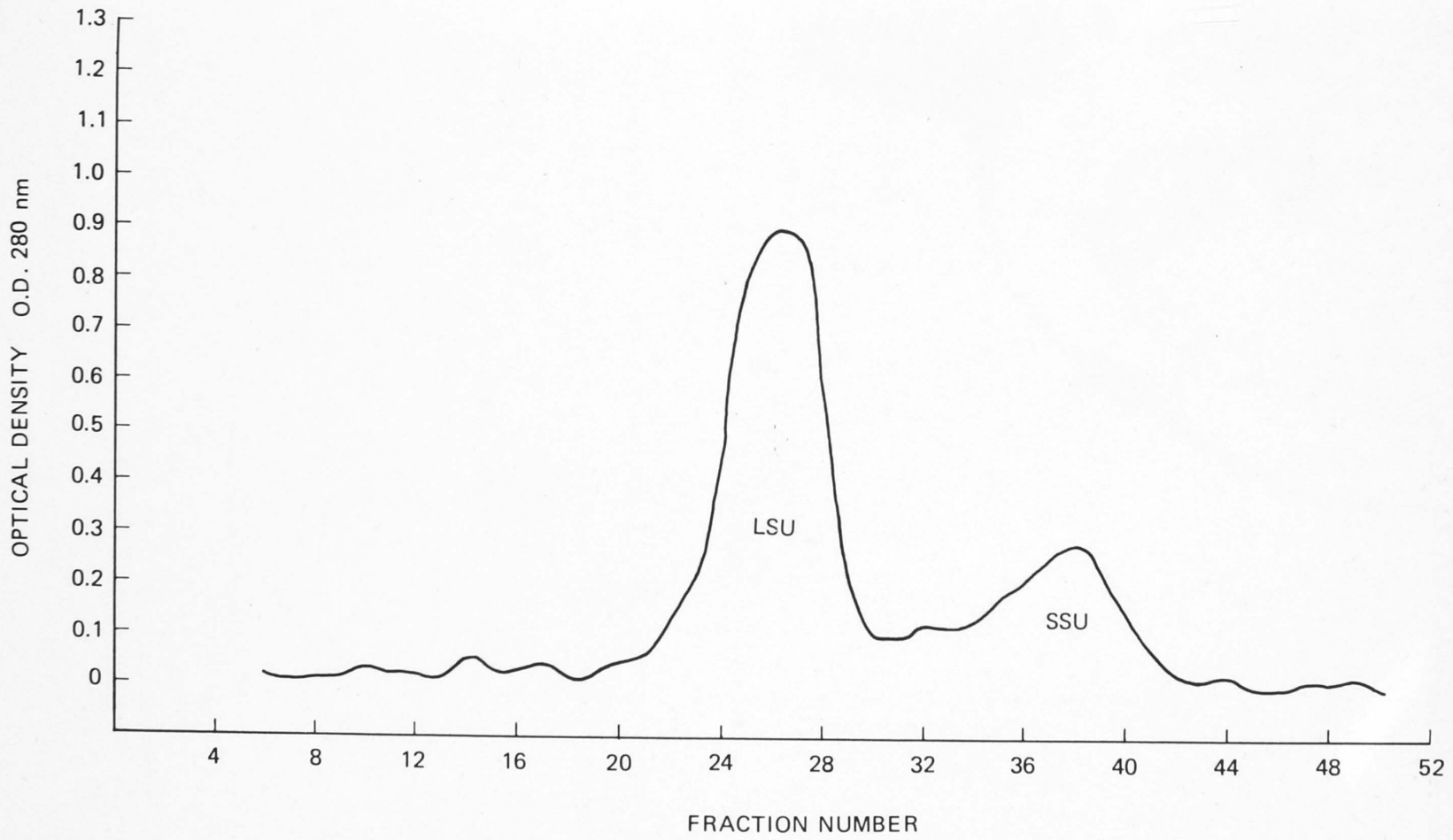
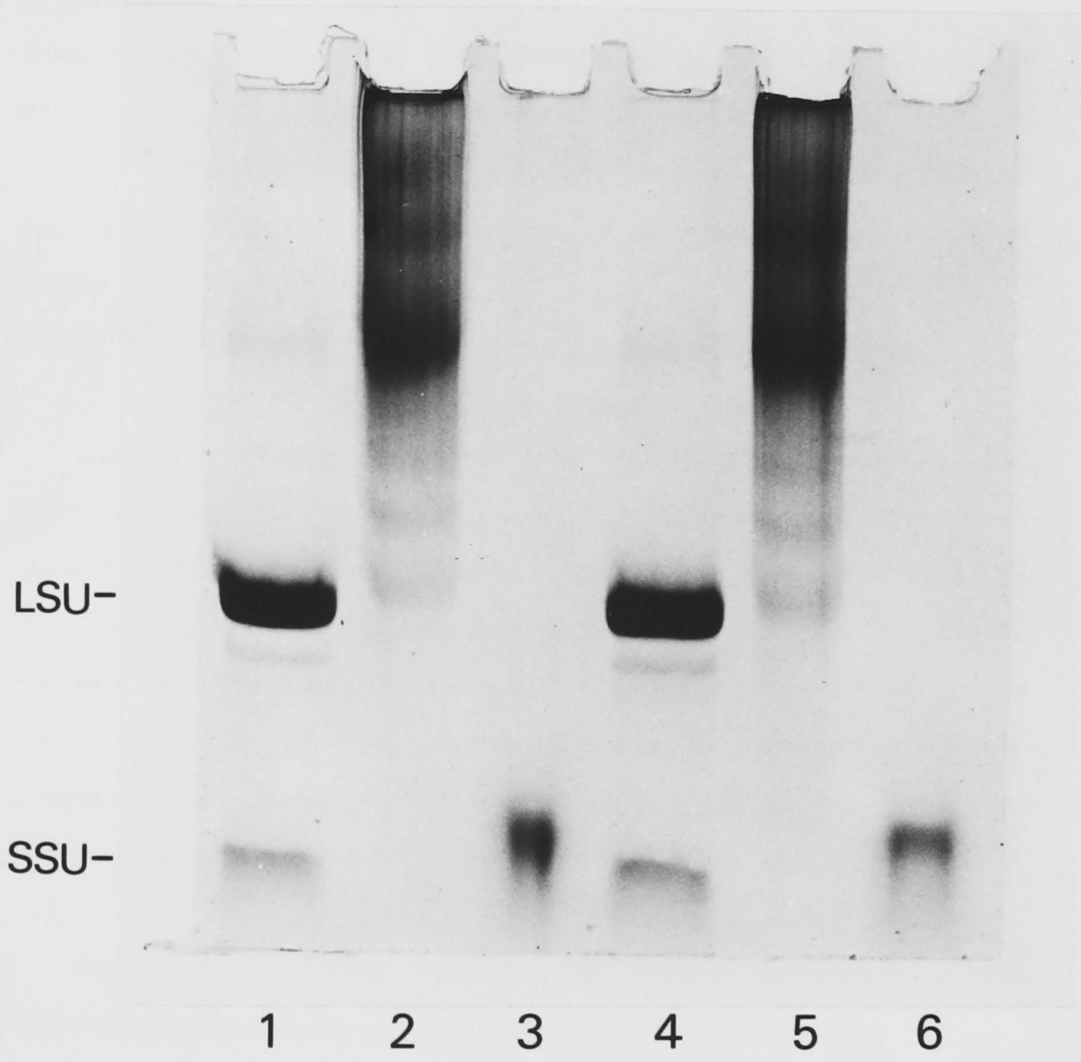

OPTICAL DENSITY O.D. 280 nm
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
LSU
SSU
4
8
12
16
20
24
28
32
36
40
44
48
52
FRACTION NUMBER

Fig. 3.12. SDS-gel electrophoretograms of LSU and SSU polypeptides separated by G100 chromatography.

RuBPC-ase of HR and MF genotypes was isolated by Sepharose 6B column chromatography (Chapter 2.4.2). The LSU and SSU polypeptides of RuBPC-ase were separated by G100 chromatography. 50 µg protein of each sample was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% w/v) and heated at 50°C for 10 min. The sample was loaded on a 13% Davis gel. Electrophoresis was carried out for 2 h at 30 mA and approx. 120 V. at room temperature. The gel was stained in Coomassie Blue. Lane 1: RuPBC-ase of HR, lane 2: LSU of HR; lane 3: SSU of HR, lane 4: RuBPC-ase of MF, lane 5: LSU of MF, lane 6: SSU of MF.


LSU-
SSU-
1
2
3
4
5
6

of the RuBPC-ase. The SSU (lane 3) of HR has one band similar to that of SSU band of HR RuBPC-ase. The LSU and SSU polypeptides of RuBPC-ase of the MF genotype are in lane 4. The LSU of the MF genotype (lane 5) has the same multiple bands of six as the LSU of HR (lane 2). The SSU of MF (lane 6) appears in a single band. It can be concluded that SDS treatment following isolation by G-100 segregated the molecules of LSU into six high molecular weight bands (lanes 2 and 5).

3.2.2. (iv) Isoelectric Focusing of RuBPC-ase, LSU, and SSU from HR,HP, and MF Genotypes of Medicago

Samples of RuBPC-ase of HR, HP, and MF plant genotypes were collected from the elution fractions of Sepharose 6B. They were further purified on a DE-52 column and after S-carboxymethylation they were applied to an isoelectric focusing gel. RuBPC-ase of HR, HP and MF (lane 1,2,3) have three LSU bands located between pI 6.5 and 6.9 on the gel (Fig. 3.13). HR and HP genotypes have one (appears to be double on this gel) at pI 7.5 and MF has one visible polypeptide above the LSU bands, on the alkaline side of the gel (pI 7.9).

Fig. 3.14 shows the isoelectric focusing of RuBPC-ase of the three genotypes and the purified SSU of HR and MF genotypes.

The pattern of isoelectric focusing of RuBPC-ase is the same in Fig. 3.14 as in Fig. 3.13, but SSU of HR shows two polypeptides and SSU of MF shows two polypeptides. Both genotypes have a band which is masked under an LSU band where the RuBPC-ase was focused. The HR band has a pI of 6.8, and that of MF has pI 6.9.

The isoelectric focusing of the purified SSU (without S-carboxylation) of the three genotypes is shown in Fig. 3.15. HR

Fig. 3.13. Isoelectric focusing separation of RuBPC-ase of HR, HP and MF (lanes 1,2,3) genotypes of Medicago.

The same methods and materials were used in all three experiments presented in Fig. 3.13, Fig. 3.14, Fig. 3.15.

The isoelectric focusing apparatus, preparation of gel slabs, and S-carboxymethylation of RuBPC-ase are described in greater detail in Chapter 2.8.1, 2.8.2. and 2.8.3.

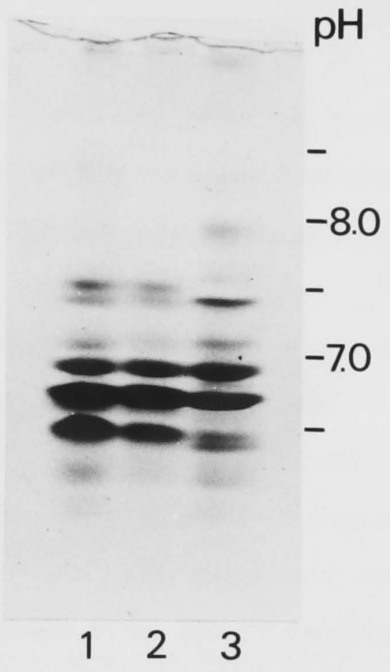
The RuBPC-ase of HR, HP and MF genotypes was separated by Sepharose 6B column chromatography. The SSU of the three genotypes were isolated by Sephadex 100.

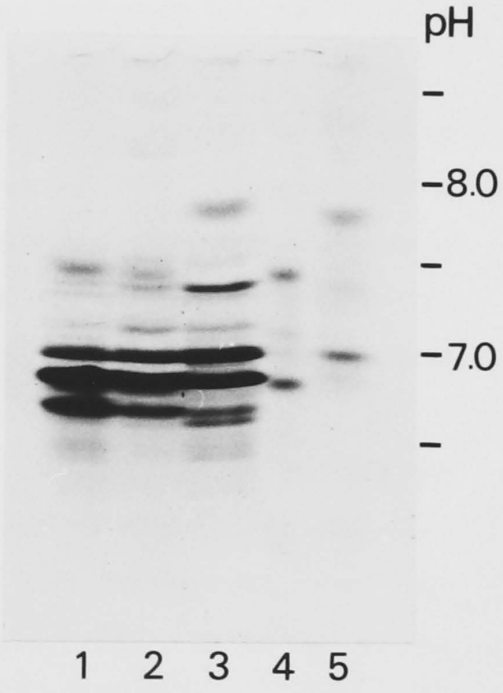
Twice-recrystallized urea and dithiothreitol were added to protein solutions which were then incubated for 30 min at room temperature.

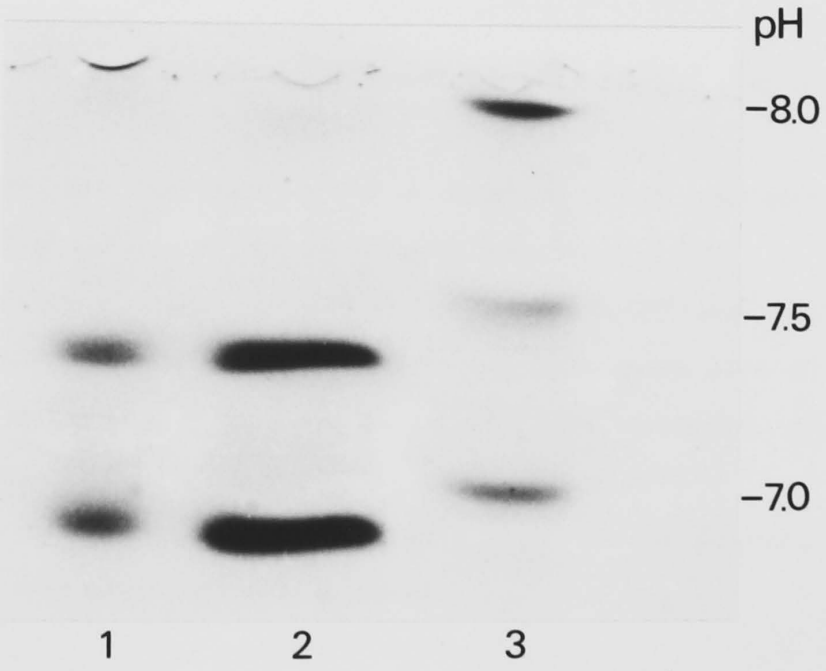
Solutions containing 40 µg protein were loaded on compartments of the isoelectric focusing gel. The apparatus was set initially to 80 V, 6 mA, with power kept constant below 1 W for the 9 h duration of the experiment. The gels were stained with 0.04% bromophenol blue in a solution containing ethanol, glacial acetic acid, and water, for 4 hours.

Fig. 3.14. Isoelectric focusing separation of RuBPC-ase of HR, HP, and MF genotypes (lanes 1,2,3) and SSU of HR, MF genotypes (lanes 4,5).

Fig. 3.15. Isoelectric focusing separation of SSU of HR, HP and MF genotypes. (lanes 1,2,3).


pH
–
–8.0
–
–7.0
–
1 2 3


pH
–
–8.0
–
–7.0
–
1 2 3 4 5


pH
–8.0
–7.5
–7.0
1 2 3

and HP genotypes have two identical polypeptides, at pI 6.8 and 7.4, whereas MF has three polypeptides, at pI 6.9, 7.5 and 8.0.

3.2.3. Tryptic peptide maps of LSU and SSU of HR and MF genotypes of Medicago

The tryptic peptide maps of LSU of HR and MF genotypes of Medicago are presented in Fig. 3.16 and Fig. 3.17. Fingerprinting of trypsin hydrolysates of the polypeptides by two dimensional chromatography and electrophoresis showed 37 peptides in both LSU peptide maps. The experiment failed to differentiate between the LSU polypeptides of HR and MF genotypes.

The tryptic hydrolysates of SSU polypeptides of HR and MF genotypes (Fig. 3.18, Fig. 3.19) showed 19 peptides on each map. In contrast to the tryptic maps of LSU polypeptides, each SSU polypeptide contained at least one unique peptide, as marked by the arrows.

3.3. DISCUSSION

The main features of the experiments described in this chapter are as follows:

(1) RuBPC-ase and "cytoplasmic" proteins were well separated and were free of contamination by phenolic substance.

(2) The amino acid composition of both RuBPC-ase and "cytoplasmic" proteins showed that essential amino acids were all present at sufficient concentration, with the exception of methionine.

(3) The newly designed IEF apparatus eliminated the problems associated with other isoelectric equipment.

Fig. 3.16. Tryptic peptide map of LSU polypeptides of HR genotype.

The LSU and SSU of RuBPC-ase were separated by Sephadex G100, dialyzed against water, and freeze dried overnight. They were subjected to trypsin hydrolysis and the tryptic peptides were resolved by two-dimensional solvent chromatography and paper electrophoresis by the same technique (Fig. 3.16 and Fig. 3.17) described previously (Kung et al. 1972).

Fig. 3.17. Tryptic peptide map of LSU polypeptide of MF genotype.

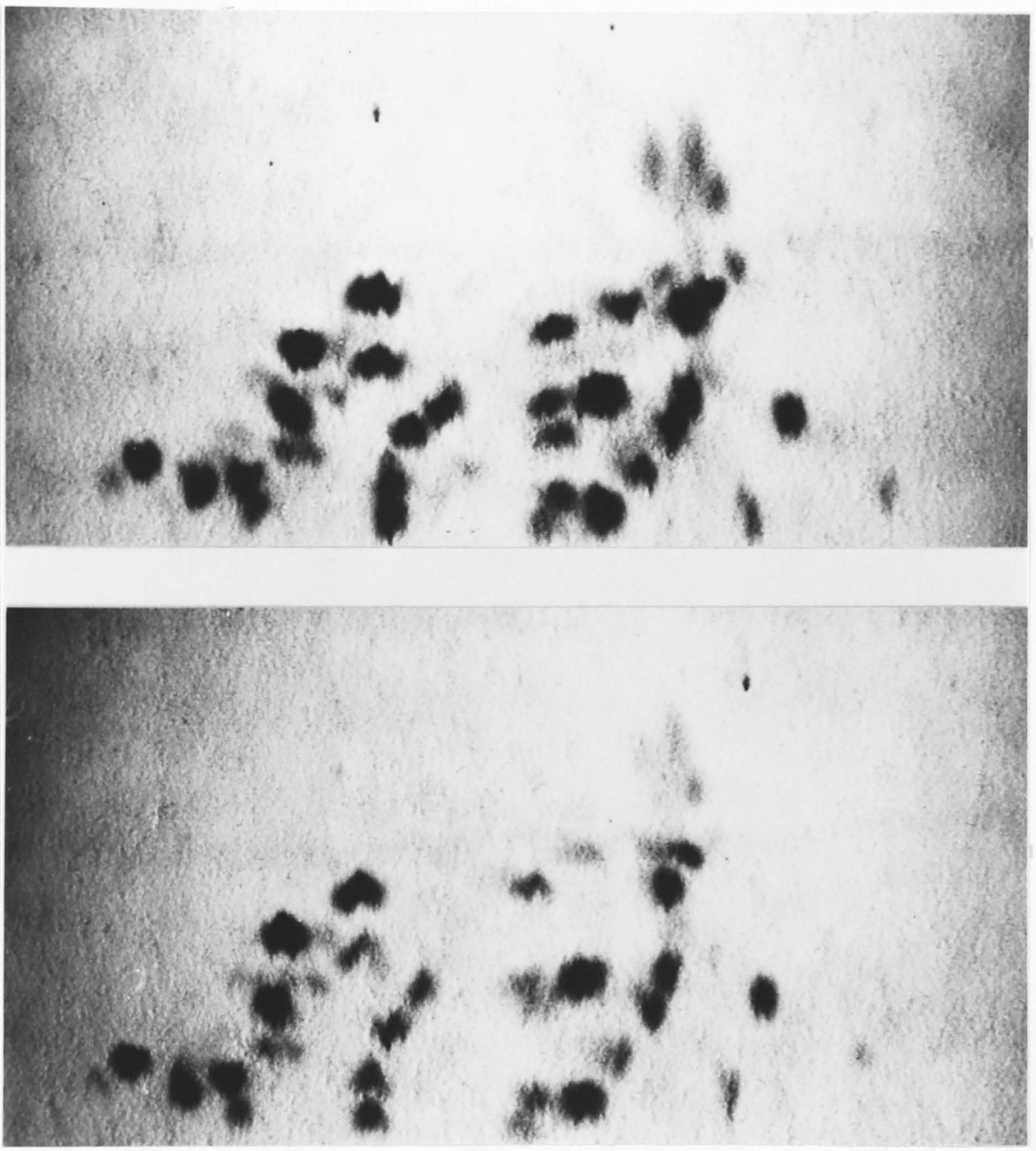
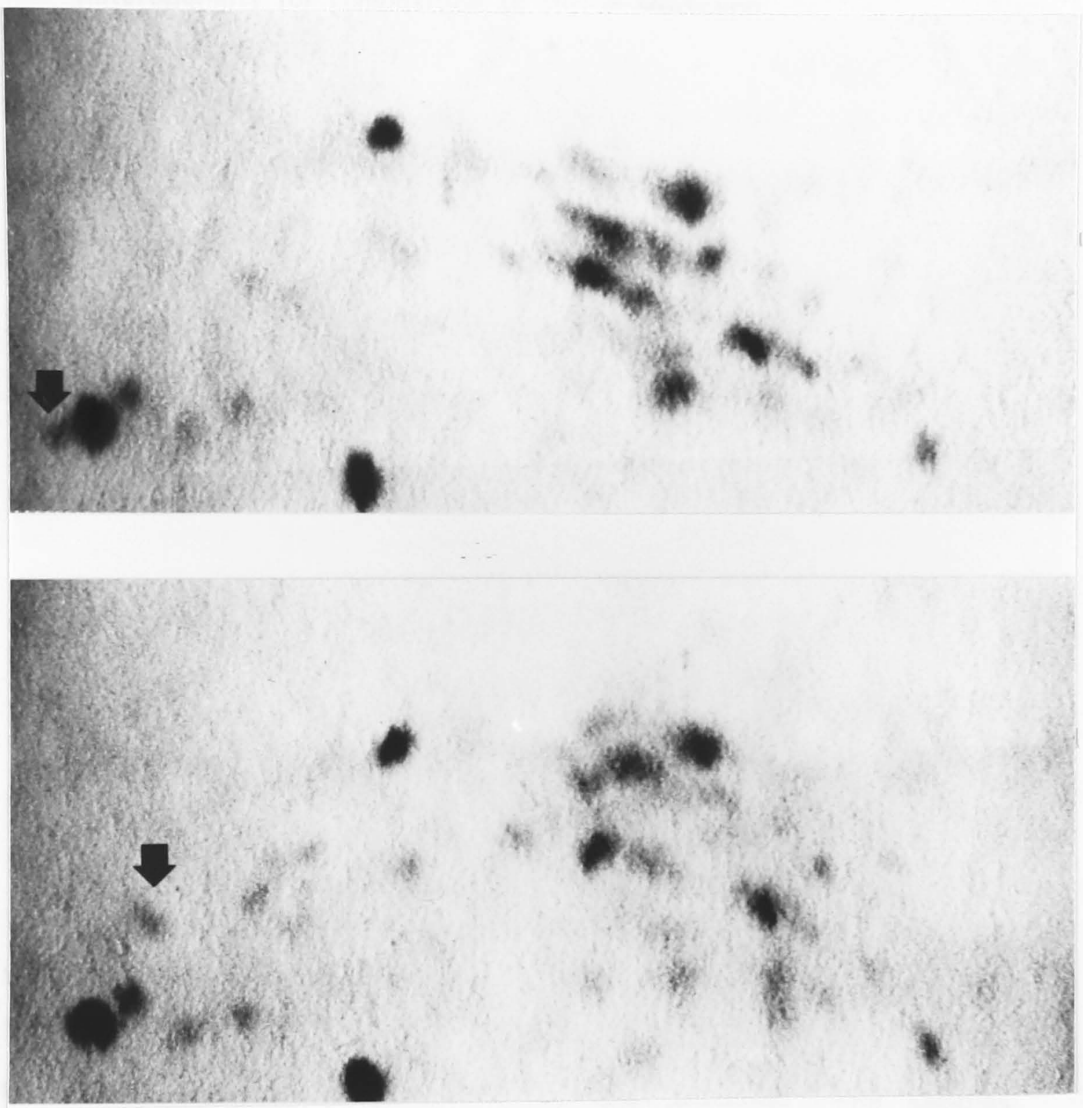


Fig. 3.18. Tryptic peptide map of SSU polypeptides of HR Medicago genotype.

After separation by Sephadex G100 chromatography, the LSU and SSU of RuBPC-ase were subjected to trypsin hydrolysis and the tryptic peptides resolved by two dimensional solvent chromatography and paper electrophoresis by the same technique described previously (Kung et al. 1972). Techniques used in Fig. 3.18 and Fig. 3.19 are the same as for Fig. 3.16 and 3.17.

Fig. 3.19. Tryptic peptide map of SSU polypeptides of MF Medicago genotype.



(4) The isoelectric focusing of RuBPC-ase of Medicago showed that all SSU polypeptides are alkaline and demonstrated heterogeneity for composition of SSU in Medicago.

(5) Although the tryptic peptide maps of LSU were identical, unique peptides were noted in the SSU maps of HR and MF genotypes.

A major problem in the isolation of leaf proteins (including those of Medicago) for basic and applied research involves difficulties in separation of phenolic and other molecules from proteins. These contaminants made the isolated protein preparations coloured (from light brown to dark green) and extracts had leafy or bitter flavours. There are numerous publications concerning this topic. Bray et al. (1978) studied the effectiveness of various solvents and solvent mixtures in removing pigments from the leaf protein concentrate prepared from M. sativa, and obtained a hot-air-dried product which was neither black nor had a hard texture. Sixteen solvent systems representing polar, non-polar, polar + Non-polar (1:1 v/v), and the usual lipid solvents (chloroform : methanol, 2:1 v/v, and ethanol : diethyl ether, 3:1 v/v) were evaluated. Acetone, ethanol, propan-2-ol, and butan-1-ol were found to be most effective. In further tests, propan-2-ol and butan-1-ol emerged as the most promising solvents for extraction of leaf proteins. The samples extracted with these solvents had pale green colour and a bland to slightly leafy flavour.

The present experiments showed that gel filtration (Sepharose 6B, Sephadex G25) removes the small molecules which contaminate the leaf proteins. The products RuBPC-ase and "cytoplasmic" protein, are pure, white and tasteless.

The amino acid composition of proteins has great nutritional significance. Seed storage proteins are frequently deficient in essential amino acids. In terms of human nutrition the limiting amino acid in all of the major cereals, cotton, and peanuts is lysine. The other limiting amino acid in corn is tryptophan and in rice and wheat it is threonine. Peanut protein is deficient in lysine, methionine and threonine (Oram and Brock, 1972). The essential amino acids of leaf proteins of Medicago are all available in the required concentration except methionine, which is slightly below the recommended amount.

Isoelectric focusing (IEF) of proteins has been carried out using several types of apparatus (cf. Righetti and Drysdale, 1976; Righetti and Gianazza, 1980). These include the commercially produced LKB flat-bed apparatus, the disc electrophoresis apparatus of Davis (1964) as modified for IEF use by Wrigley (1968), and the vertical flat sheet apparatus described by Reid and Bieleski (1968). Choosing an apparatus suitable for the analysis of RuBPC-ase from plant leaves presents some difficulties. With the flat bed apparatus the upper surface of the gel is exposed to the air, resulting in oxidation of the enzyme and preventing formation of clear bands. Also, when direct comparison of banding patterns of RuBPC-ase from different sources is required, the disc method is of limited value because it is preferable to make such comparisons on the same gel under identical conditions. The vertical flat sheet apparatus provides a solution to the problems encountered with the two previous types, but the large buffer container associated with such an apparatus could result in an uneven pH gradient in the gel, again making cross comparison of closely related substances difficult.

The modified apparatus which I have described in this thesis overcomes the difficulties associated with the three types previously mentioned, when applied to the IEF analysis of RuBPC-ase and other oxidizable plant proteins.

Isoelectric focusing of RuBPC-ase also provides a suitable phenotypic marker for plant species. Chen et al., (1977) determined the relative position of the isoelectric bands of LSU and SSU of RuBPC-ase from different species of plants. They found three LSU and two acidic SSU polypeptides in spinach and three LSU and one acidic SSU polypeptide in M. sativa. My results are in agreement as far as the LSU and SSU polypeptide composition of spinach is concerned, but M. sativa was found to have two alkaline SSU polypeptides. The HR and HP genotypes have two SSU polypeptides with identical pI values, while the MF genotype has two or three SSU polypeptides with different pI values. This result reflects genetic differences between M. sativa and M. falcata.

Tryptic peptide analysis appears to be a useful way to compare LSU and SSU of various genotypes and species and to determine the degree of heterogeneity of subunits. Kawashima et al. (1971) showed that tryptic peptides of the LSU of five Nicotiana RuBPC-ase's are very similar. In contrast to the LSU, significant differences were found in the composition of some tryptic peptides of the SSU among the same five species of Nicotiana. These findings are similar to the results given here for Medicago. The LSU of HR and MF genotypes were similar and there was one peptide difference between SSU composition of HR and MF genotypes.

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CHAPTER 4.

ISOLATION AND ANALYSIS OF RuBPC-ase IN MEDICAGO, SPINACEA AND NICOTIANA SPECIES

4.1. INTRODUCTION

S-carboxymethylated crystalline RuBPC-ase of Nicotiana tabacum cv. Turkish Samsun has been dissociated and separated into three LSU polypeptides and two SSU polypeptides by isoelectric focusing in polyacrylamide gels containing 8 M urea (Gray et al., 1978). The three LSU were clustered near pH 6.3, and the two SSU polypeptides were near pH 5.5. The three LSU polypeptides resolved by isoelectric focusing could not be differentiated by amino acid analysis or by fingerprinting of trypsin hydrolysates or chymotrypsin hydrolysates of the individual polypeptides. The two SSU polypeptides, resolved by hydroxyapatite chromatography in 0.1% sodium dodecyl sulphate as well as by isoelectric focusing, were shown to be distinct polypeptides. The two polypeptides were found to have different tyrosine : tryptophane ratios as shown by ultraviolet spectra in 0.1 M NaOH, different tyrosine contents as shown by amino acid analysis, and different peptide fingerprints after trypsin hydrolysis.

Gray et al., also found that proteolytic degradation during the purification procedure was not responsible for multiple LSU polypeptide bands. However, when the protein was isolated from older leaves without protection from polyphenols the electrofocusing pattern frequently showed the presence of additional intermediate bands. Iodoacetamide, used for preparation of RuBPC-ase before isoelectric focusing, did not produce any change in the pattern of LSU polypeptides. Their experimental results indicated that the LSU

polypeptide pattern was not the result of artifacts during the preparation or analysis of protein. Therefore they concluded that the two SSU polypeptides are separate gene products, but that the three LSU polypeptides represent either three separate gene products or modifications of a single gene product.

Phenotypic variation of RuBPC-ase in the plant kingdom was also investigated by Chen et al. (1977). The polypeptide composition of RuBPC-ase showed that all plant species examined had three LSU polypeptides separated by about 0.1 pH unit from each other. The number of SSU were found to be variable.

Wildman (1979) studied the evolution of RuBPC-ase in the genus Nicotiana, comprising 66 species, and 20 different kinds of RuBPC-ase molecules were identified. With respect to LSU, the genus could be separated into four different groups which differ in isoelectric point of their polypeptides. All species have three S-carboxymethylated LSU polypeptides. He also found a range of differences in the isoelectric points of the SSU polypeptides.

In contrast to the above investigations, an analysis of the subunits of RuBPC-ase of Triticum aestivum by gel isoelectric focusing in 8 M urea (O'Connell and Brady, 1981) revealed one LSU and one SSU. Carbamidomethylation of the RuBPC-ase before isoelectric focusing, using a 300-fold molar excess of iodoacetamide over protein thiol groups, resulted in three bands of LSU. Preparative procedures involving aggregation of the RuBPC-ase also resulted in complex isoelectric focusing patterns. The simplest patterns, with one type of LSU and one type of SSU, were obtained when the RuBPC-ase was isolated rapidly and gently by immunoprecipitation or preparative polyacrylamide gel electrophoresis

and analysed by isoelectric focusing without alkylation of thiol groups.

Genetic analysis showed in Nicotiana that in reciprocal hybrids the isoelectric points of the three LSU polypeptides correspond to the isoelectric points of the polypeptides of the female parent. The genetic determinants for the three LSU polypeptides are inherited and expressed together; there is no separation of the inheritance of individual polypeptides. These findings indicated that genes which code for the LSU are inherited only from the maternal line and therefore are located in chloroplast DNA.

Genetic analysis of the coding information for the SSU of RuBPC-ase also demonstrated that the SSU polypeptide characteristics of both parents of Nicotiana were found in each reciprocal N. glauca x N. tabacum F_1 hybrid. Therefore, each parent contributed genetic information for the SSU, i.e., both parents contributed pollen DNA (nuclear DNA) (Kawashima and Wildman 1972; Chan and Wildman 1972; Sakano et al., 1974).

The experiments of O'Connell and Brady (1981), were restricted to one species, Triticum aestivum. The present investigation therefore reexamined the validity of their finding concerning only one LSU polypeptide in other plant species. Firstly, the polypeptide composition of RuBPC-ase of HR and MF genotypes of Medicago was established, followed by analysis of the amino acids. Secondly, the polypeptide composition of RuBPC-ase of Medicago, Spinacea, and Nicotiana, was investigated. Thirdly, isoelectric focusing of RuBPC-ase of N. glutinosa, N. tabacum and N. excellsior was carried out and the polypeptide composition examined.

4.2. RESULTS

4.2.1. Isolation of RuPBC-ase by 6% Davis Gel Electrophoresis

Leaf extract was applied to 6% Davis gel and gel electrophoresis was carried out for 4 h at 4°C. The gel was removed from the glass frame after electrophoresis and examined in a Chromatavue ultraviolet cabinet. The RuBPC-ase was concentrated about 1 cm below the origin of the gel as indicated by a light band (Fig. 4.1.). Similar experiments were done using a separate 6% Davis gel. After electrophoresis the gel was placed in Coomassie blue solution for approximately 4 h (Fig. 4.2). The bands were visible after overnight destaining. The RuBPC-ase was stained as a dark band about 1 cm below the origin. Several minor bands which migrated further on the gel were the "cytoplasmic" proteins of the plant extracts. Both methods were utilized for rapid identification of RuBPC-ase.

4.2.2. Separation of LSU and SSU by 13% Davis Gel Electrophoresis

The RuBPC-ase band of Medicago HR genotype was cut out from the 6% Davis gel, homogenized, and after extraction by SDS tube electrophoresis the RuBPC-ase solution was applied to a 13% Davis-SDS gel. RuBPC-ase was separated (Fig. 4.3) into LSU (53 kd) and SSU (14 kd) polypeptides.

4.2.3. Determination of Purity of LSU and SSU by SDS Gel Electrophoresis

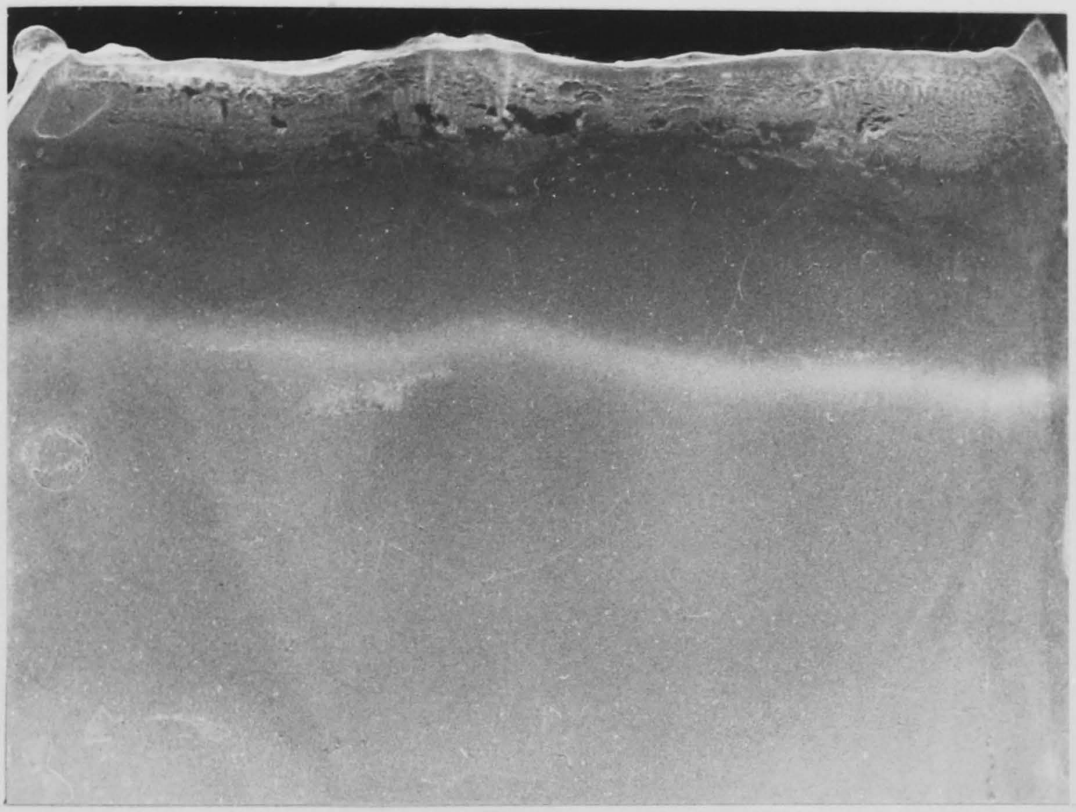
The LSU and SSU polypeptides were extracted from the 13% Davis gel and tested for purity of protein composition. The isolated LSU and SSU polypeptides and a sample of RuBPC-ase of Medicago HR genotype were applied to another 13% Davis gel. The amount of

Fig. 4.1. Electrophoretogram of RuBPC-ase of Medicago HR genotype leaf extract. Identification was by UV light. The RuBPC-ase is seen as a light band near the center of the gel.

The gel electrophoresis procedure was identical for both Fig. 4.1 and 4.2.

Three grams of fresh leaf material were homogenized in 3 ml of extracting buffer (Tris 200 mM, Na_2 EDTA 5 mM, $MgSO_4 \cdot 7H_2O$ 25 mM, polyvinylpyrrolidone 1%, 2-mercaptoethanol 10 mM, cysteine 5 mM, adjusted to pH 8.5 with boric acid) in a precooled mortar (4°C). The homogenate was squeezed through a layer of Miracloth. The filtrate was centrifuged at 108,000 g for 45 min. 6% Davis gel was prepared as described in Chapter 2.5.1. The electrophoresis buffer contained Tris 45 mM, boric acid 25 mM, and Na_2 EDTA 0.7 mM, pH 8.5. 0.7 ml of plant extract was applied to a 6% Davis slab gel which was run at 30 mA and approximately 120V for 4h.

Fig. 4.2. Electrophoretogram of RuBPC-ase of Medicago HR genotype leaf extract by 6% Davis gel electrophoresis. Identification was by Coomassie blue stain.



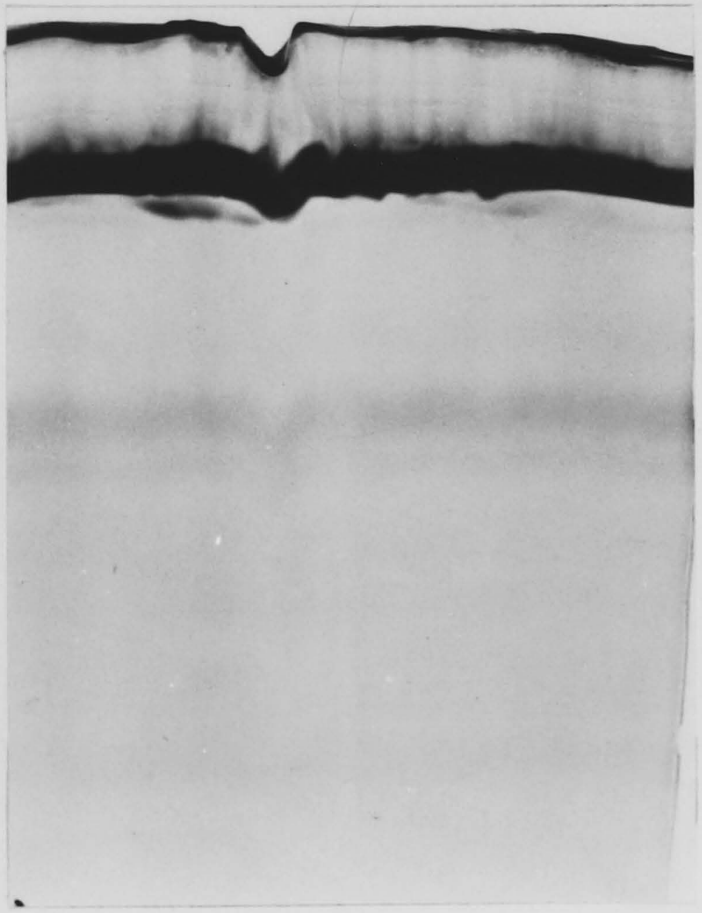
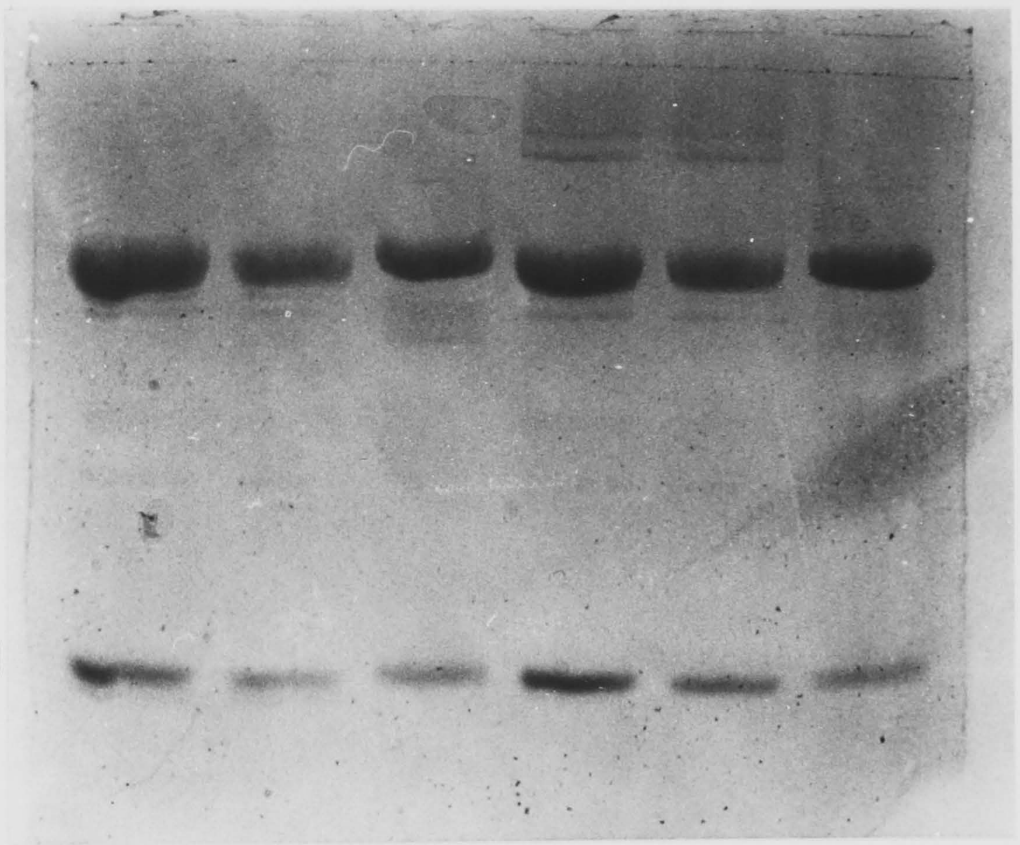


Fig. 4.3. SDS gel electrophoretograms of RuBPC-ase of Medicago HR genotype, by 13% Davis gel.

The RuBPC-ase was previously isolated by 6% Davis gel. The band containing the enzyme was cut out from the gel slab, and the homogenized gel was placed in a tube of an electrophoresis tank as described in Chapter 2.5.5. After electrophoresis for 4 h at 1 mA per tube, the RuBPC-ase was eluted, supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% w/v), then heated at 50°C for 10 min. This sample was loaded on a Davis 13% Davis gel (either with or without divisions at the origin of the gel), and electrophoresis was carried out for 2 h at 30 mA and approx. 120v at room temperature. The gel was stained in Coomassie Blue. All six lanes represent the same sample

53 kd = LSU and 14 kd = SSU


53 Kd
14 Kd

protein was 50 µg per sample of RuBPC-ase, LSU or SSU polypeptides. The SDS gel electrophoresis was carried out for 2 h at 30 mA and approximately 120 V at room temperature. The gel was stained in Coomassie blue. Fig. 4.4. shows two RuBPC-ase bands, at 53 kd and at 14 kd (lane 1). LSU polypeptide (l ane 2) had a single band at 53 kd and no other bands appeared. The SSU polypeptide (lane 3) had one band at 14 kd. Therefore it can be concluded that the isolated LSU and SSU polypeptides from 13% Davis gel were not significantly contaminated with other proteins.

4.2.4. Isolectric Focusing of RuBPC-ase and SSU of HR and MF Genotypes

RuBPC-ase was extracted from 6% Davis gel and SSU of HR and MF genotypes were obtained from 13% Davis gel following electrophoresis. 40 µg of protein from each sample was applied to the isoelectric focusing gel without any S-carboxymethylation. After a 9 h IEF run the gels were stained in bromophenol blue.

The results of an isoelectric focusing run are illustrated (2-replications) in Fig. 4.5 . RuBPC-ase of HR genotype (lane 1) showed one LSU and two SSU polypeptides. The LSU band is located at p1 7.0; one SSU band is just below the LSU and the second SSU was found between pl 7.5 and 8.0. The two bands of the isolated SSU HR polypeptide (lane 2) corresponded to the positions of SSU of RuBPC-ase in lane 1. The RuBPC-ase of MF genotype (lane 3) had one LSU band (pl 7.0) and two SSU polypeptide bands at pl 8 and pl 8.5. However, the purified SSU of MF genotype had three polypeptide bands (lane 4 - at pl 7, pl 8, and pl 8.5). The SSU at pl 7 was masked by the LSU band of RuBPC-ase (lane 3).

Fig. 4.4. SDS gel electrophoretograms of RuBPC-ase, LSU and SSU proteins of Medicago HR genotype.

SDS gel (13%) was prepared as described in Chapter 2.5.3. RuBPC-ase was previously extracted from 6% Davis gel by tube electrophoresis (Chapter 2.5.5.), without SDS at 4°C. The LSU and SSU polypeptides had also been previously extracted from a 13% SDS Davis gel using SDS tube electrophoresis (Chapter 2.5.5.) at room temperature. 50 µg each of RuBPC-ase, LSU and SSU polypeptides were added to SDS (2% w/v) plus 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, then loaded on a 13% SDS Davis slab gel. The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx. 120 V at room temperature. The gel was stained in Coomassie Blue solution.

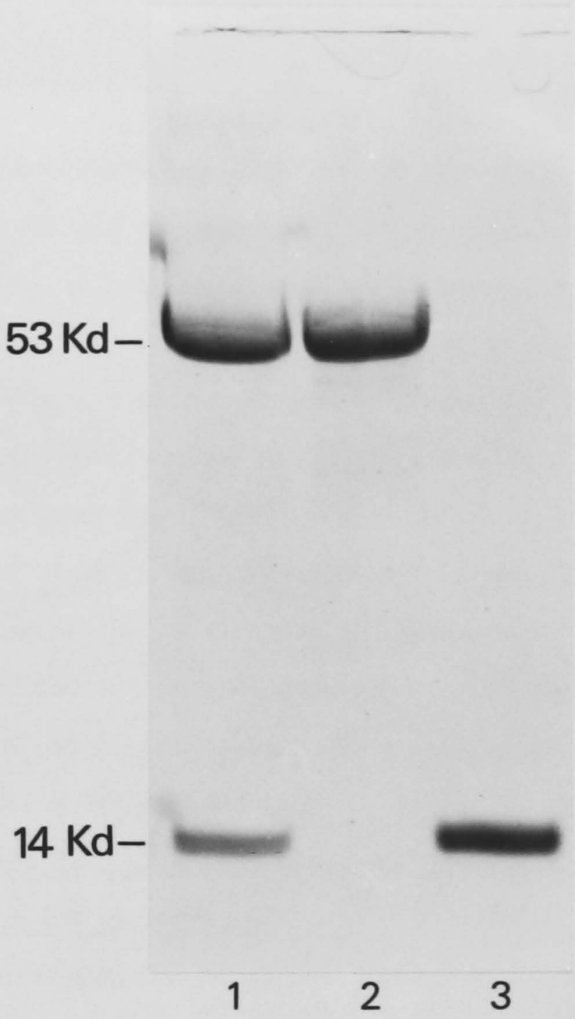
Lane 1 : RuBPC-ase; lane 2 : LSU; lane 3 : SSU; 53 kd = LSU, 14 kd = SSU.

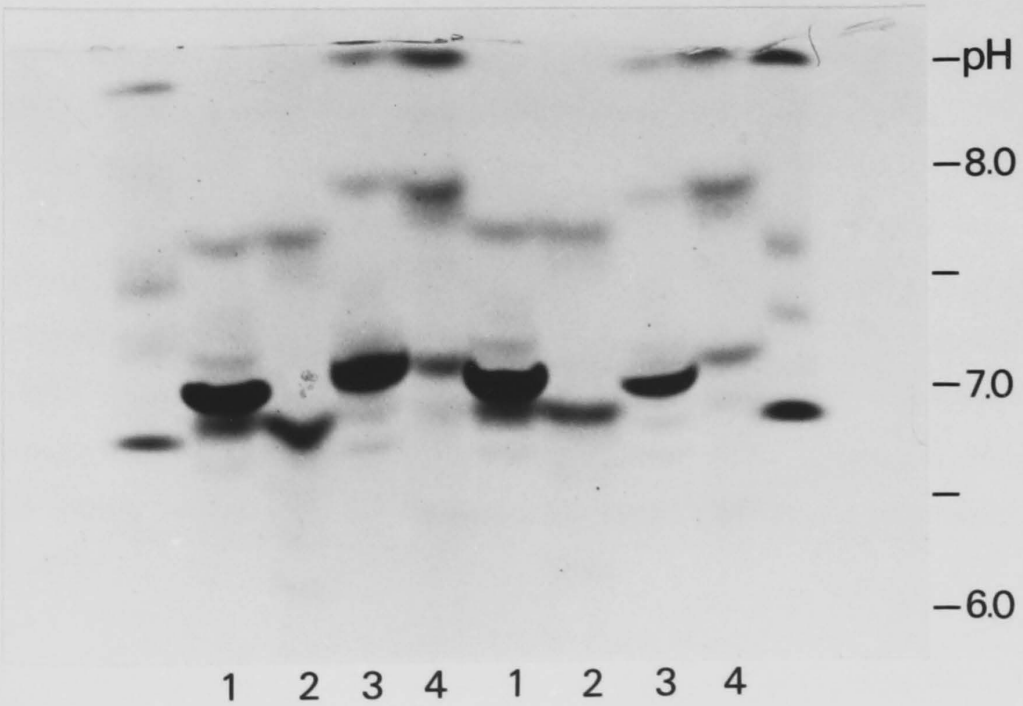
Fig. 4.5. Photograph of isoelectric focusing separation of RuBPC-ase and SSU of Medicago HR and MF genotypes.

Samples of RuBPC-ase were previously extracted from 6% Davis gel (Chapter 2.5.1.) and the samples of SSU polypeptides were extracted from 13% Davis gel (Chapter 2.5.3.), using tube electrophoresis as described in Chapter 2.5.5.

40 µg of each sample was supplemented with 20 mg of 2x crystallized urea plus 1 mg of dithiothreitol and the mixture was incubated for 30 min at room temperature. The experiment was carried out without carboxymethylation.

The apparatus and preparation of the gel slab were described in Chapter 2.8.1. and 2.8.2., respectively. Sample compartments of the isoelectric focusing gel were loaded with the protein samples (two replications) as follows : lane 1: RuBPC-ase of HR genotype; lane 2: SSU polypeptide of HR genotype; lane 3: RuBPC-ase of MF genotype; lane 4: purified SSU polypeptide of MF genotype. The two outside lanes represented marker molecules (Chapter 2.2). The apparatus was set initially at 80 V and 6 mA, with power being kept constant at 1.0 W for the duration of the experiment. The gel was normally pre-run for 15-30 minutes while the running time for the experiment was 9 h at 4°C. Gels were stained for 4 h with 0.04% bromophenol blue solution containing ethanol, glacial acetic acid, and water (10:1:9), then destained with a 6:1:13 mixture of the same solvents.


53 Kd–
14 Kd–
1
2
3


–pH
–8.0
–
–7.0
–
–6.0
1 2 3 4 1 2 3 4

The densitometric scans (600 nm) of isoelectric focusing gels of RuBPC-ase of HR and MF genotypes are presented in Fig. 4.6. The LSU polypeptides of both genotypes are located 2.5 - 3.0 cm from the origin of the gel. The first SSU polypeptide of the HR genotype is located at 1.75 cm, and the second SSU migrated 2.80 cm from the origin. The SSU polypeptides of the MF genotype are located at 0.3, 1.4 and 2.7 cm from the origin. The ratio of LSU/SSU polypeptides is 3.91:1 and 3.77:1 for HR and MF genotypes. This result demonstrates again the differences in number and position of SSU polypeptides of the HR and MF genotypes. It is also noted that the LSU/SSU (total SSU) polypeptide ratios are close to the expected (based on molecular weight) 4:1 ratio.

4.2.5. Amino Acid Composition of RuBPC-ase, LSU and SSU of Medicago

Amino acid analyses were carried out on the RuBPC-ase LSU and SSU of the HR, HP and MF Medicago genotypes and the results are presented in Table 4.1. The most common amino acids of RuBPC-ase were glutamic acid, leucine, aspartic acid, arginine, lysine, alanine and phenylalanine. The amino acid composition of the LSU of the three genotypes differed very little. In contrast, the amino acid composition of the SSU of the three genotypes varied considerably. The MF genotype differed from that of HR and HP, particularly in the proportions of lysine, arginine, aspartic acid, serine and glutamic acid. Statistical analysis confirmed a significantly greater variability among the SSU than among the LSU of these three genotypes, the variability of residues per 100 residues being 3.88 times greater ($P < 0.001$).

Fig. 4.6. Densitometric scans of isoelectric focusing gels for RuPBC-ase of Medicago HR and MF genotypes.

The procedure for isolation and preparation of RuPBC-ase, as well as the isolectric focusing of both RuPBC-ase and SSU polypeptides is described in Chapter 2.8.

The stained gels were cut in a vertical direction and the slices (lanes) were scanned with a Gilford scanning spectrophotometer (600 nm).

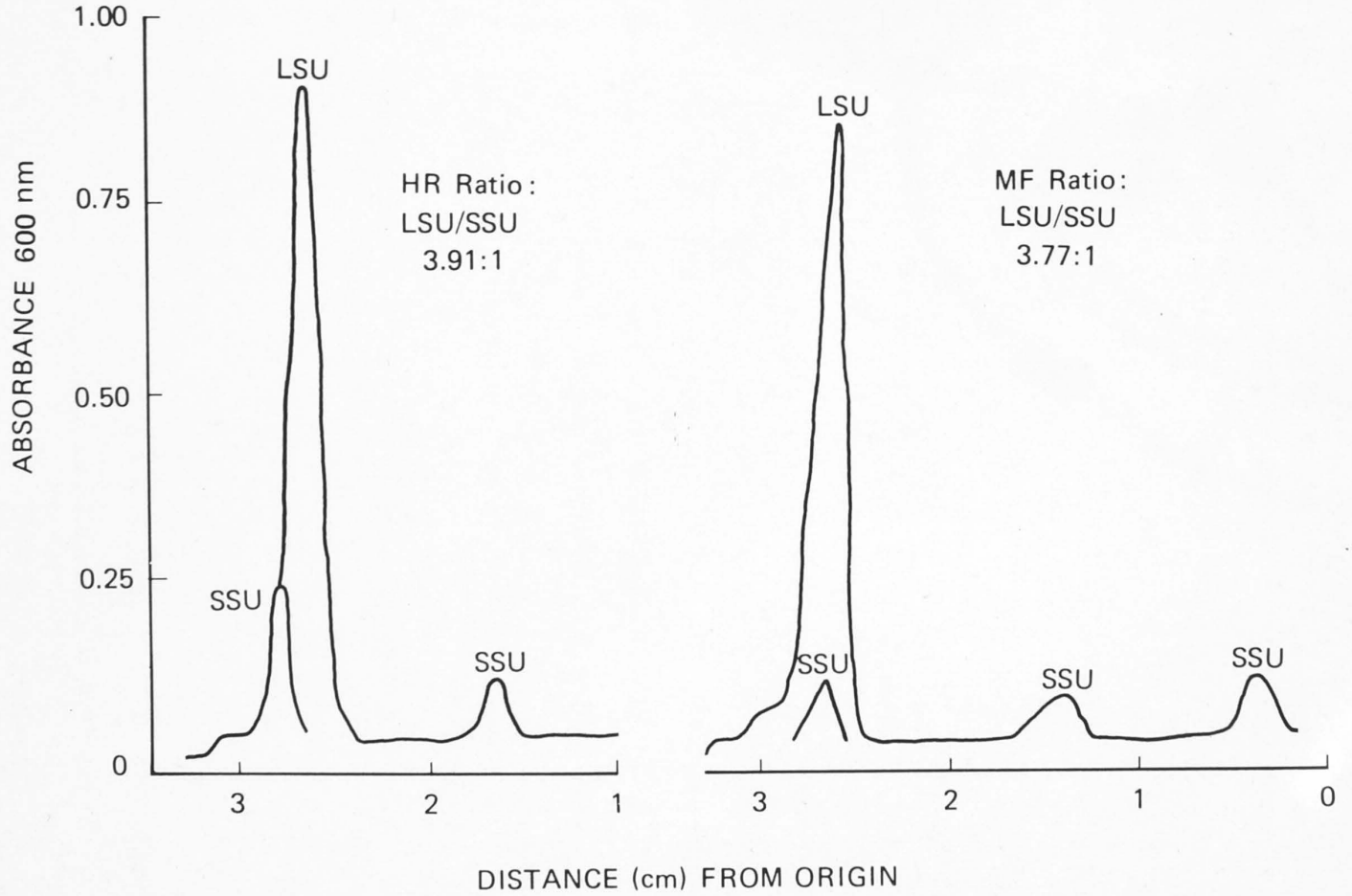

1.00
0.75
0.50
0.25
0
ABSORBANCE 600 nm
LSU
SSU
SSU
HR Ratio:
LSU/SSU
3.91:1
LSU
SSU
SSU
SSU
MF Ratio:
LSU/SSU
3.77:1
3
2
1
3
2
1
0
DISTANCE (cm) FROM ORIGIN

Table 4.1. Amino acid composition of RuBPC-ase from the Medicago HR genoytpe and of LSU and SSU from HR, HP and MF genotypes.

RuBPC-ase was isolated by Sepharose 6B column chromatography (Chapter 2.4.2). LSU and SSU were isolated by SDS gel (13%) electrophoresis (Chapter 2.5.3). Amino acid compositions were determined by the method of Byers (1971).

Table 4.1. Amino acid composition of RuBPC-ase from the Medicago HR genotype, and of LSU and SSU from HR, HP and MF genotypes

	RuBPC-ase	Large Subunit (LSU)			Small Subunit (SSU)		
		HR	HP	MF	HR	HP	MF
		Residues per 100			Residues per 100		
Lysine	6.97	6.07	5.91	6.17	9.17	8.27	7.65
Histidine	3.78	4.36	4.21	3.99	2.51	2.40	1.91
Arginine	8.94	8.68	7.77	8.46	8.45	7.23	6.70
Aspartic acid	9.32	9.78	10.10	9.48	6.68	6.83	7.57
Threonine	5.85	5.85	5.59	5.23	3.99	4.15	4.54
Serine	3.53	2.55	2.57	2.86	3.54	3.60	4.26
Glutamic acid	11.97	10.08	10.18	10.32	14.81	14.81	13.53
Proline	2.82	3.61	3.52	3.74	5.83	5.80	5.92
Glycine	5.49	6.19	5.89	5.90	3.79	3.85	4.03
Alanine	6.39	7.43	7.19	7.50	3.35	3.35	3.73
Valine	5.83	6.78	6.70	6.30	5.08	5.46	5.51
Methionine	2.22	2.44	2.36	2.31	0.95	1.02	1.20
Isoleucine	5.04	3.93	5.11	5.30	5.91	6.01	6.17
Leucine	9.73	9.76	10.55	10.44	10.15	10.42	10.54
Tyrosine	5.69	5.07	5.32	5.12	8.19	9.21	8.71
Phenylalanine	6.43	6.79	7.09	6.84	7.61	7.59	8.03

4.2.6. Inheritance of Subunits of RuBPC-ase in Medicago

Subunits of RuBPC-ase of HR and MF genotypes and their hybrids HR x MF and MF x HR were compared by isoelectric focusing, as shown in Fig. 4.7.

The purified RuBPC-ase samples, previously obtained from 6% Davis gel, were applied (two replications) to an isoelectric focusing gel. Lane 1 shows the subunit composition of RuBPC-ase of the HR genotype. The one LSU polypeptide is at pI 7.0; there are two SSU polypeptides, one located just below the LSU and the other at approximately pI 7.7. The RuBPC-ase of MF (lane 4) has one LSU at pI 7.0 and two visible SSU at pI 7.9 and 8.5. The reciprocal hybrids, of HR x MF and MF x HR, are in lane 2 and 3 respectively. The hybrids have an identical polypeptide composition. Apart from a single LSU at pI 7.0, there are four visible SSU's which represent the composition of the SSU of HR and MF genotypes. The results show that both parents and their hybrids have one single LSU at the same pI value. Analysis of coding information for SSU of the reciprocal hybrids indicated that the genes of both HR and MF parents are transmitted by the pollen and the information for SSU is located in nuclear DNA.

4.2.7. SDS Gel Electrophoresis of LSU and SSU Extracted from an Isoelectric Focusing Gel

The aim of this experiment was to determine the identity of the LSU and SSU bands from an isoelectric focusing gel. The gel pieces of LSU and SSU bands were homogenized and applied to a 13% SDS Davis gel. Samples analysed were the following: RuBPC-ase of the HR genotype (Fig. 4.8, lane 1); LSU and SSU of HR genotype (lanes

Fig. 4.7. Isoelectric focusing separation of RuBPC-ase of HR and MF genotypes and of their hybrids.

The four samples of RuBPC-ase were originally isolated from 6% Davis gel (Chapter 2.5.1) by means of tube electrophoresis (Chapter 2.5.5.). 40 µg of RuBPC-ase of each sample was supplemented with 20 mg 2x-recrystallized urea plus 1 mg dithiothreitol and the mixture was incubated for 30 min at room temperature. The experiment was carried out without S-carboxymethylation.

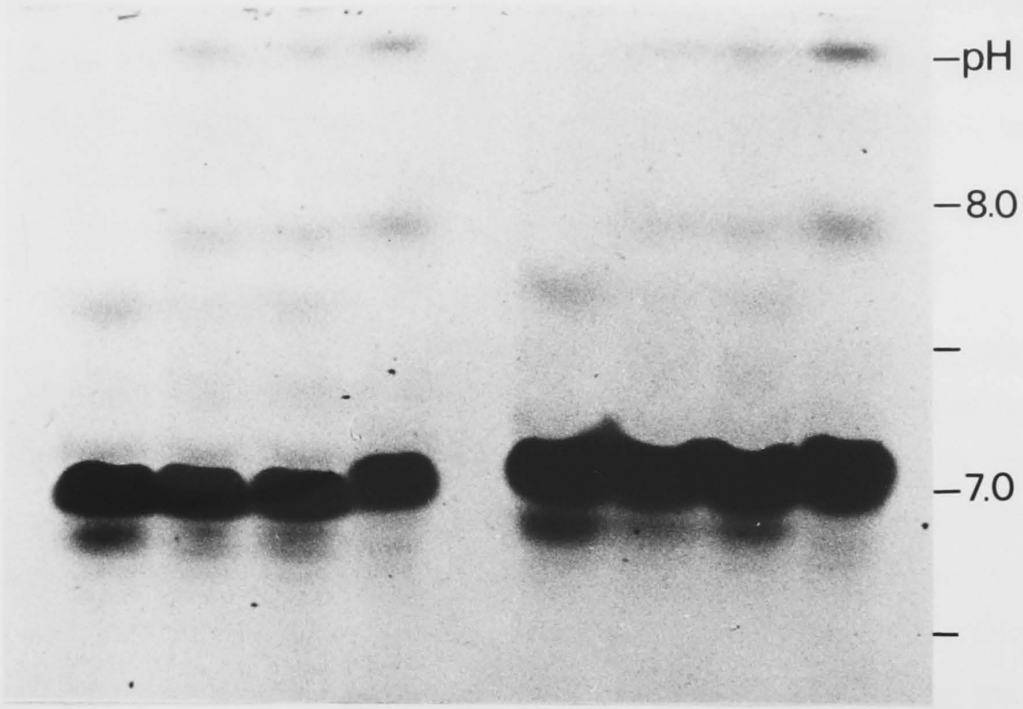
The apparatus and preparation of the isoelectric focusing gel were described in Chapter 2.8.1 and 2.8.2, respectively. The apparatus was set initially to 80 V, 6 mA with the power being kept constant at 1.0 W for the duration of the experiment. The gel was normally pre-run for 15-30 minutes, while the running time was 9 h. Gels were stained for 4 h in 0.04% bromophenol blue solution and destained with ethanol, glacial acetic acid and water (6:1:13) solution.

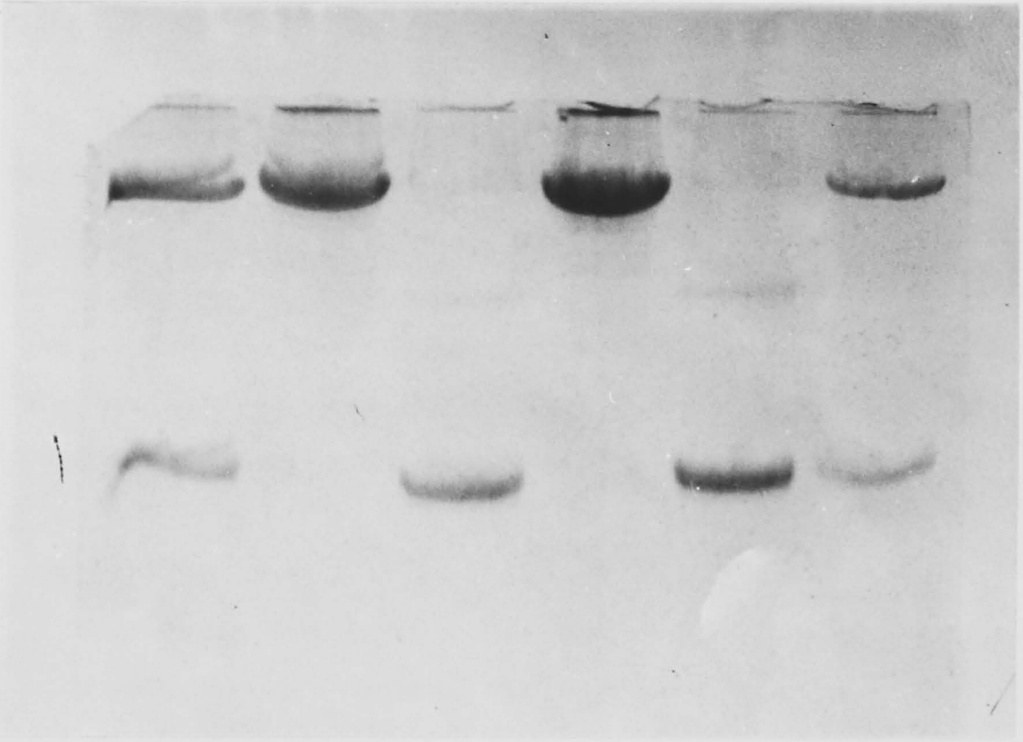
Lane 1: RuBPC-ase of HR, lane 2: RuBPC-ase of HR x MF, lane 3: RuBPC-ase of MF x HR, lane 4: RuBPC-ase of MF.

Fig. 4.8. SDS gel electrophoretograms of LSU and SSU extracted from an isoelectric focusing gel.

Samples of RuBPC-ase of HR and MF genotypes were previously extracted from 6% Davis gel (Chapter 2.5.1). The LSU and SSU polypeptides were similarly extracted from an isoelectric focusing gel (Fig. 4.5). The gel pieces were homogenized in a 1 ml syringe and applied to the origin of a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx. 120 V at room temperature. The gel was stained in Coomassie Blue solution.

Lane 1: RuBPC-ase of HR, lane 2: LSU of HR, lane 3: SSU of HR, lane 4: LSU of MF, lane 5: SSU of MF, lane 6: RuBPC-ase of MF.


—pH
—8.0
—
—7.0
—
1 2 3 4 1 2 3 4


1 2 3 4 5 6

2 and 3); LSU and SSU of MF genotype (lane 4 and 5); RuBPC-ase of the MF genotype (lane 6). All IEF-extracted samples proved to be pure LSU and SSU polypeptides.

4.2.8. Isoelectric Focusing of RuBPC-ase from Medicago, Spinacea and Nicotiana

Samples of RuBPC-ase of Medicago (HR genotype) Spinacea, and Nicotiana were extracted from a 6% Davis gel and applied to an isoelectric focusing gel in three replications (Fig. 4.9). The HR genotype (lane 1) had one LSU (heavy band) and two small SSU polypeptides. Spinacea (lane 2) had one LSU (heavy band)and one acid SSU polypeptide. Nicotiana (lane 3) had one LSU and two SSU polypeptides which migrated to the more acidic region of the gel. The most important finding is that all three species had a single LSU polypeptide with different pI values.

4.2.9. Isoelectric Focusing of RuBPC-ase from N. glutinosa, N. tabacum and N. excellsior

The polypeptide composition of three Nicotiana species is illustrated in Fig. 4.10 (2 replications of each sample or lane). N. glutinosa (lane 1) has one LSU and two acidic SSU which migrated very close to each other. N. tabacum (lane 2) also shown one LSU and two well separated SSU polypeptides. N. excellsior (lane 3) shows also one LSU and four acidic SSU polypeptides. The LSU (heavy bands) of the three species are separated by 0.1 unit of pI.

4.3. DISCUSSION

The results of Chapter 4 demonstrated that pure LSU and SSU polypeptides can be isolated by 13% Davis gel electrophorsis. The HR and MF genotypes each have a single LSU polypeptide. Heterogeneity

Fig. 4.9. Isoelectric focusing separation of RuBPC-ase of Medicago, Spinacea and Nicotiana.

RuBPC-ase of the above species was isolated by the method described in Chapter 2.5.1, using 6% Davis gel. The enzymes were extracted from the gels by tube electrophoresis, without SDS (Chapter 2.5.5). 40 µg of each RuBPC-ase sample was supplemented with twice-crystallized urea and dithiothreitol and the mixtures were incubated for 30 min at room temperature. The preparation was carried out without S-carboxymethylation.

The apparatus and preparation of gel were described in Chapter 2.8.1 and 2.8.2, respectively. The samples were loaded on the isoelectric focusing gel in three replications. The apparatus was set initially to 80 V, 6 mA, with the power kept constant at 1.0 W for the duration of the experiment. The gel was normally pre-run for 15-30 min, while the running time was 9 h. The gels were stained for 4 h in 0.04% bromophenol blue solution, and destained with ethanol, glacial acetic acid, and water (6:1:13) solution.

Lane 1: RuPBC-ase of Medicago, lane: RuPBC-ase of Spinachea, lane 3: RuBPC-ase of Nicotiana.

Fig. 4.10. Isoelectric focusing separation of RuBPC-ase of N. glutinasa, N. tabacum, and N. excellsior. Preparation of samples and conditions for electrophoresis was as described for Fig. 4.9.

Lane 1: RuPBC-ase of N. glutinosa, lane 2: RuPBC-ase of N. tabacum, lane 3: RuPBC-ase of N. excellsior.


1 2 3 1 2 3 1 2 3
1 2 3 1 2 3

of the SSU was observed by gel electrophoresis and further supported by amino acid analysis. Genetic analysis of SSU polypeptides of Medicago confirmed that synthesis of the SSU is controlled by nuclear DNA. Medicago, Spinacea and Nicotiana have one single LSU polypeptide with different pI values. Three Nicotiana species have also been found with a single LSU polypeptide.

Separation of LSU and SSU has also been achieved by G100 column chromatography (Kung, et al., 1974), but the present results (Chapter 3.2.2. iv) show further that the LSU consists of multiple bands. Separation of LSU and SSU polypeptides by 13% SDS Davis gel electrophoresis therefore seems preferable (Chapter 4.2.1.) to that of the Sepharose G100 technique.

RuBPC-ase of M. sativa has been reported (Chen et al., 1977) to have three LSU polypeptides and one acidic SSU polypeptide. The present investigation demonstrated that both M. sativa and M. falcata have only one LSU polyeptide and two to three alkaline polypeptides (Chapter 4.2.4.). This heterogeneity of SSU in Medicago was further supported by amino acid analysis (Chapter 4.2.5.). In addition, it has been found that the number of methionine residues per 100 amino acid residues of LSU and SSU of Medicago differed from that of Nicotiana. The ratio of methionine residues per 100 residues of Nicotiana (Gray et al., 1978) was the same in both LSU and SSU. In contrast, the methionine ratio for LSU polypeptides of HR, HR and MF genotypes was twice as high as for the SSU of the same three genotypes (Chapter 4.2.5.). One significance of this finding is that if the LSU and SSU of Medicago are labelled with ^{35}S the expected incorporation of isotope will be markedly different to that in Nicotiana, due to the different methionine content of the two species rather than to the LSU/SSU polypeptide ratio.

Maternal inheritance of the LSU polypeptide could not be confirmed in Medicago as it was in Nicotiana (Kung, 1976), as all the LSU polypeptides in Medicago have the same pI values (Chapter 4.2.4.). However, the genetic analysis of SSU in Medicago confirmed the nuclear DNA control (Chapter 4.2.6.) previously reported for Nicotiana (Kung, 1976).

Differences in subunit composition were also reported for Spinacea and Nicotiana. Chen et al. (1977) showed that Spinacea had three LSU and two SSU polypeptides. N. tabacum had three LSU and two SSU polypeptides (Wildman, 1979). My results demonstrated that both species have only one single LSU polypeptide (Chapter 4.2.8.), although SSU polypeptides were found to be different. Spinacea "normally" had one single SSU (Chapter 4.2.8.), but two SSU were found when the RuBPC-ase was S-carboxymethylated (Chapter 3.2.2. I).

The SSU polypeptide composition of N. glutinosa, N. tabacum and N. excellsior was the same in Wildman's (1979) experiment as in the present investigation (Chapter 4.2.9.). However, one LSU polypeptide was found in all three Nicotiana species in my experiments (Chapter 4.2.9.), in contrast to the three LSU observed by Wildman (1979) for the same species. The migration order and pI value difference (0.1) between the single LSU polypeptides in my experiments are similar to those reported by Wildman.

No correlation was found between amino acid composition and band position difference in isoelectric focusing of polypeptides, where pI value differed by only 0.1-0.2. Noguchi et al. (1978) showed that the amino acid composition of the LSU of Medicago, Spinacea and Nicotiana was identical. On the other hand 0.1 and 0.2 pI unit

differences were noted for LSU polypeptides of the three species in the present investigation (Chapter 4.2.8). Wildman (1979) found no difference between the charged (electrically) amino acid composition of N. glutinosa, N. tabacum and N. excellsier (LSU) but both his experiments and mine (Chapter 4.2.9.) demonstrated pI 0.1 and 0.2 unit differences between LSU polypeptides on isoelectric focusing gels. If differences of pI values of SSU polypeptides are large enough this difference could be reflected in the composition of charged amino acids both in Nicotiana (Wildman, 1979) and Medicago (Chapter 4.2.5.).

S-Carboxymethylation of RuBPC-ase contributed to the appearance of three LSU polypeptides. The same treatment also altered the SSU polypeptide number of Spinacea and Medicago. S-carboxylmethylated RuBPC-ase of Spinacea had two SSU polypeptides (Chen et al., 1977; Chapter 3.2.2.). In contrast, in the absence of S-carboxymethylation, Spinacea had one SSU (Chapter 4.2.1). S-carboxymethylated RuBPC-ase of MF had no SSU polypeptide at pH 8.5 gel position (Chapter 3.2.2.v) but the polypeptide was present at pI 8.5 in samples of RuBPC-ase of MF genotype which was not carboxymethylated (Chapter 4.2.3.).

Evidence for one LSU polypeptide was also supported by the finding that only one gene for the LSU exists in the chloroplast genome in Pisum sativum (Bedbrook et al., 1979). It seems reasonable to postulate that the one-LSU polypeptide of RuBPC-ase is the general rule in higher plants.

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CHAPTER 5.

QUANTITATIVE GENETICS OF LEAF PROTEINS AND CHLOROPLAST DEVELOPMENT IN LEAVES OF MEDICAGO

5.1 INTRODUCTION

Genetic investigations of soluble protein content in Medicago have been well documented in scientific publications during the last two decades. Gegenbach (1971) studied the heritability of protein level (percent protein) and yield by using three clones differing in protein level and obtained data from the S_o, F_1, S_1 and F_2 generations. Narrow sense heritability estimates from regression of F_2 on F_1 were h^2 = 51% for protein level and 23% for protein yield. Other broad-sense estimates of heritability for protein percentage range from 26% to 60% (Gegenbach, 1971). Heinrichs (1972) used an 8-clone diallel population to study the heritability of soluble RuBPC-ase protein by variance component methods and obtained a heritability value of 33% for RuBPC-ase. Using a population of 60 clones in a replicated nursery, Miltimore et al. (1974) found a broad-sense heritability value of 28% for RuBPC-ase. This was somewhat lower than the 46% previously reported (Heinrichs and Miltimore, 1970). Narrow-sense heritability estimates were obtained for soluble RuBPC-ase and "cytoplasmic" protein and total soluble protein concentrations in M. sativa (Gutek et al. 1976). Heritability estimates (h^2) were based on the parent-offspring regression method using data from 32 unselected clones and their open-pollinated progenies. The h^2 estimates for the soluble proteins for 1972 and 1973 were as follows: soluble RuBPC-ase (19 and 20%), soluble "cytoplasmic" fraction (19 and 27%), total soluble protein (23 and 31%). Although these heritability estimates are relatively low, it should still be possible to lower the soluble protein content of M. sativa by

consecutive cycles of recurrent selection procedures involving well-replicated progeny tests.

Lamprecht et al., (1965) conducted an experiment consisting of ten plants from South African M. sativa which were used in a diallel experiment design to provide estimates of breeding values and genetic parameters. The inbreds, single crosses and clones were replicated four times in a randomized design. Although the nitrogen content of herbage had a high (56%) heritability, it was negatively correlated with all yield components, so expected selection gains were considerably reduced as far as nitrogen content is concerned. The relationship between yield and nitrogen content of the herbage, however, was curvilinear and among the progeny of selected individuals some plants occurred which were both rich in nitrogen and high yielding. The results therefore indicated that considerable improvement in yield of good quality herbage is obtainable by rather simple breeding procedures. Individual plants growing under full competition may be selected.

The influence of polyploidization on RuBPC-ase, buffer-soluble protein, chlorophyll, and DNA was examined in fully expanded leaves of isogenic diploid-tetraploid (2x-4x) and tetraploid-octaploid (4x-8x) sets of M. sativa (Meyers et al. 1982a). Activity of RuBPC-ase (expressed per mg protein or per mg chlorophyll), leaf tissue concentration of RuBPC-ase, buffer soluble protein, chlorophyll, and DNA were similar for different ploidy levels of the 2x-4x set. Tetraploids and octaploids were similar in RuBPC-ase activity (expressed per mg protein or per mg chlorophyll) and in leaf tissue concentrations of RuBPC-ase and DNA. Octaploids were significantly lower than tetraploids in concentrations of chlorophyll and

buffer-soluble protein. When compared on a per leaf basis, tetraploids were 80% higher than comparable diploids in buffer-soluble protein and had essentially twice the fresh weight, RuBPC-ase, chlorophyll, and DNA. Ratios of RuBPC-ase to DNA and chlorophyll to DNA were similar across ploidy levels of both isogenic sets, suggesting that the cellular content of chlorophyll and RuBPC-ase increased proportionately with the amount of DNA per cell.

Photosynthetically-active protoplasts isolated from isogenic sets of diploid-tetraploid and tetraploid-octaploid M. sativa leaves were used to investigate the consequences of polyploidization in several aspects related to photosynthesis at a cellular level (Molin et al., 1982). Protoplasts from the tetraploid population contained twice the amount of DNA, RuBPC-ase, chlorophyll, and chloroplasts per cell as compared with protoplasts from the diploid population. Although protoplasts from the octaploid population contained nearly twice the number of chloroplasts and amount of chlorophyll per cell as tetraploid protoplasts, the amount of DNA and RuBPC-ase per octaploid cell was only 50% higher than in protoplasts from tetraploid populations. The rate of $C0_2$-dependent 0_2 evolution in protoplasts nearly doubled with an increase in ploidy from the diploid to the tetraploid level, but increased only 67% with an increase in ploidy from the tetraploid to octaploid level. Whereas leaves and protoplasts had similar increases in RuBPC-ase, DNA, and chlorophyll with increase in ploidy level, it was concluded that increased cell volume rather than increased cell number per leaf was responsible for the increase in leaf size with ploidy.

The influence of polyploidization on chloroplast coupling factor "1" was examined (Meyers et al., 1982b) in leaves and leaf protoplasts from isogenic diploid-tetraploid (2x-4x) and tetraploid-octaploid (4x-8x) sets of M. sativa. Coupling factor

constitutes about 10% of the thylakoid-membrane protein and is the extrinsic-membrane-protein component of the ATP-synthase complex which couples proton flux across thylakoid membranes to the synthesis of ATP. The chloroplast coupling factor was purified to homogeneity and found to contain five subunits with molecular weight of 57 kd, 54 kd, 38 kd, 210 kd, and 15 kd. Ratios of coupling factor 1 to chlorophyll of leaf protoplasts and leaves were similar for diploids and tetraploids. In the 4x-8x set, octaploid protoplasts were 90% higher in chlorophyll than were comparable tetraploids, whereas octaploids were 50 to 60% higher than tetraploids in fresh weight per cell and cellular content of coupling factor 1 and DNA. Ratios of coupling factor 1 to DNA in protoplasts were similar across ploidy levels for leaves of both isogenic sets. Therefore, the cellular content of coupling factor 1 increases proportionally with the amount of DNA per cell or gene dosage.

The above investigations showed that the synthesis of both RuBPC-ase and "cytoplasmic" proteins is under genetic control and that the total soluble protein, RuBPC-ase and chlorophyll increased with increasing amount of DNA.

The aim of the present investigation is to study the response to selection for high and low leaf protein, and the effect of selection on cell and chloroplast morphology.

5.2 RESULTS

5.2.1. Response to High and Low Protein Selection of a Lucerne Population During Three Selection Cycles.

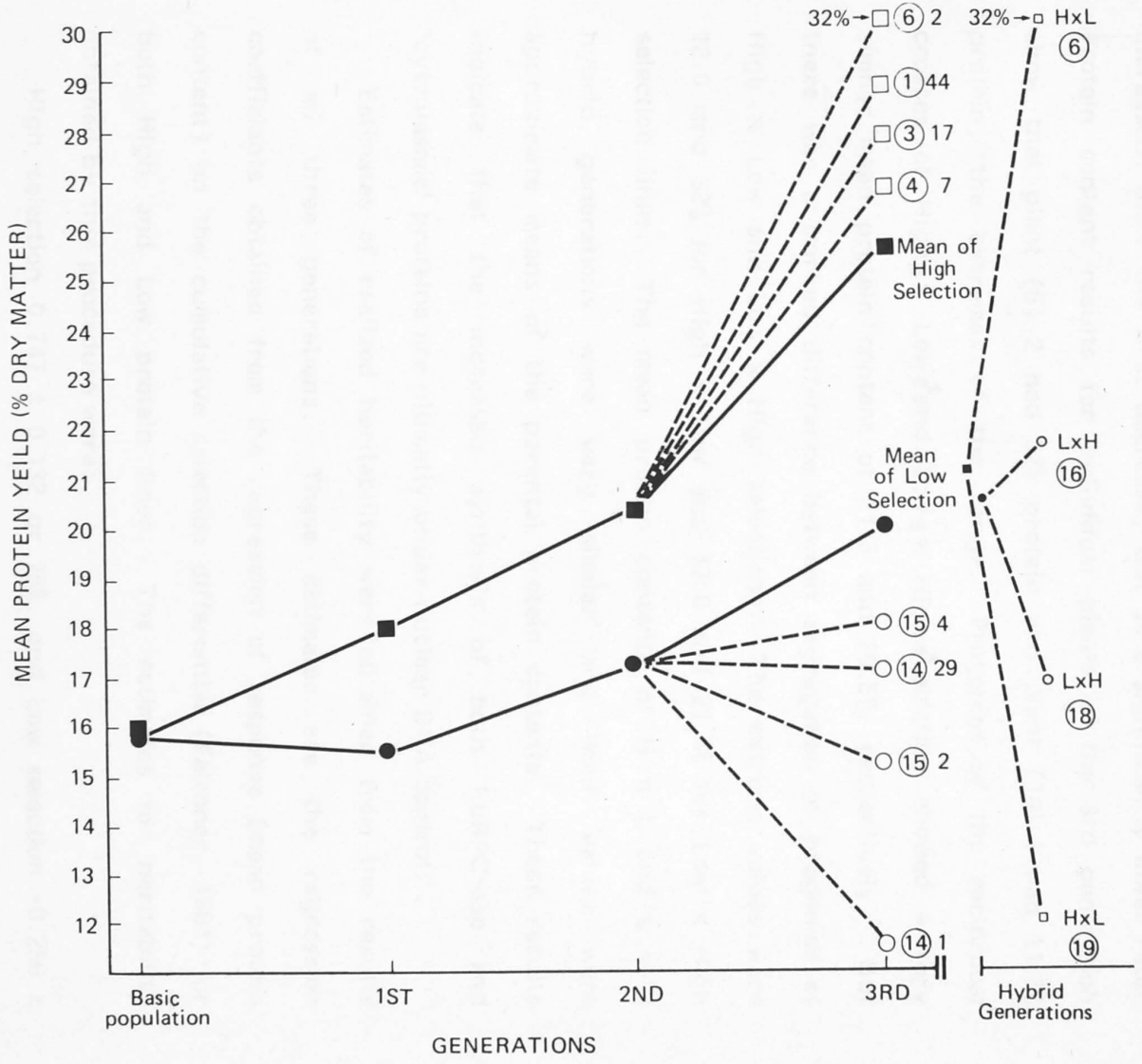
Previous publications concerning the genetic analysis of RuBPC-ase and "cytoplasmic" proteins (described in the Introduction to Chapter 5) established various magnitudes of heritability values

(broad and narrow-sense) in M. sativa, without any extensive demonstration of the response to selection. Since the heritabilities reported were rather variable, the heritability value of leaf protein content of M. sativa was reexamined. The most reliable type of analysis is the "realised heritability" (Falconer, 1964) analysis, based on the values obtained from both parent and offspring. The experiment was carried out during three generations. Later the extreme 'high' and 'low', 'low' and 'high' protein genotypes were intercrossed and the protein content of the progenies was analysed.

The results for protein yields of three generations and a hybrid generation are presented in Fig. 5.1. The basic population consisted of randomly selected plants of HR genotype. The mean protein yield was based on the analysis of 200 plants, which were in the second green-bud stage. The mean protein yield of the basic population was 16%. Selected high-protein-yielding individual plants were then intercrossed and the progenies of ten plants formed the population for the 1st generation "High" selection. In the same way the ten low-protein-yielding plants of the basic population were intercrossed and their progenies formed the 1st generation of the "Low" selection. The mean value of 100 plants of the 1st generation of High selection was 18.0% protein and of that of Low selection was 15.5%. 100 progenies of each of the High and Low selections of the 1st generations then provided two separate populations for the 2nd generation. The mean protein values for these populations were 20.0 and 17.3% for High and Low selections, respectively. Although the mean value of High selection increased in a linear way, there was also a slight increase in the case of Low selection lines. However, the difference between the mean of High and Low selection lines increased from 2.5% to 2.7% in the 1st and 2nd generations respectively. The procedure for the final (3rd) generation was the same as for the

Fig. 5.1. Diagram of response to selection for 'High' and 'Low' leaf protein in Medicago.

200 randomly selected plants of HR genotype were included in the basic generation. Plants were grown in pots at 25°C in a glasshouse. At the first green-bud stage all plants were cut back and at the second green-bud stage the individual plants were harvested. The total nitrogen content of individual plants was determined (Chapter 2.7) and this value was converted to total protein content of plants after correction for non-protein nitrogen. The best 6 to 10 'low' and 'high' protein-yielding plants were selected from each generation. They were intercrossed, by hand pollination, within the High or Low selections. The progenies (100-120 plants) of each selection provided the material for the next generation. Heritability was calculated according to Chapter 2.14. Closed squares and circles are population means. Open squares and circles for the third generation indicate values for individual plants. Large numbered circles and following numbers represent the identification of individual plants.


MEAN PROTEIN YEILD (% DRY MATTER)
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
32% → ⑥ 2
① 44
③ 17
④ 7
Mean of High Selection
32% → HxL ⑥
Mean of Low Selection
LxH ⑯
⑮ 4
⑭ 29
LxH ⑱
⑮ 2
⑭ 1
HxL ⑲
Basic population
1ST
2ND
3RD
Hybrid Generations
GENERATIONS

previous generations. The mean values of protein were 25.5 and 20.0% in High and Low selection lines, with a divergence of the means of 5.5% over the base population. In general, the High selections retained an upwards tendency, while the Low selections also increased, after the first decrease, but at a significantly lower rate. Protein content results for individual plants of the 3rd generation show that plant (6) 2 had 32% protein and plant (14) I had 11.5% protein, the extremes of the range. Progenies of the reciprocal crosses of High ♀ x Low ♂ and Low ♀ x High ♂ selection showed a very similar mean protein content of 21.3 and 20.5%, respectively. But there was a marked difference between segregation of progenies of High x Low and Low x High selections. The extreme values were 12.0 and 32% for High x Low and 17.0 and 21.8% for Low x High selection lines. The mean protein contents of H x L and L x H hybrid generations were very similar and their values were approximate means of the parental protein contents. These results indicate that the increased synthesis of both RuBPC-ase and 'cytoplasmic' proteins are ultimatly under nuclear DNA control.

Estimates of realized heritability were obtained from the results of all three generations. These estimates are the regression coefficients obtained from the regression of response (mean protein content) on the cumulative selection differential (Falconer, 1964) for both High and Low protein lines. The estimates for heritability obtained by this procedure were:

High selection 0.747 ± 0.132 or 75%, and Low selection -0.256 ± 0.158 or -26%.

It can be concluded that there was good response to high-protein selection. In contrast, selection for low protein should only have a slight gain on the basis of mean figures, but the extreme

'low' protein genotype (14) 1 still indicates a possibility of further reducing protein content. Increased mean values of the Low selection may be due to improved environmental conditions (fertilizer, watering) in the glasshouse. It is also interesting that progenies of High x Low crosses showed greater segregation of protein content than the progenies of Low x High crosses.

5.2.2. Non-protein and Protein Nitrogen Contents of High and Low Protein Genotypes.

Differences in the total nitrogen content of individual plants of M. sativa could be due either to protein nitrogen or to non-protein nitrogen. Therefore the proportions of non-protein nitrogen in 'high' and 'low' protein plants were determined (Table 5.1). Four 'high' and four 'low' protein plants were selected for this analysis. The total nitrogen (mg/100 mg), non-protein nitrogen (mg/100 mg) and proportion of non-protein nitrogen (% of total nitrogen) were determined. Considerable differences were found between the means of the total nitrogen of 'high' and 'low' protein plants. The amount of non-protein nitrogen was greater in 'high' than in 'low' protein genotypes. The means of non-protein nitrogen and of total nitrogen in both 'high' and 'low' genotypes were not statistically different. These results can be interpreted to mean that increase in total nitrogen of the leaves was due to the increase of protein nitrogen in M. sativa.

5.2.3. Ratio of RuBPC-ase and "Cytoplasmic" Proteins in 'High' and'Low' Protein Genotypes.

Selection experiments (Chapter 5.2.1) resulted in genotypes with extremes of high and low protein content. One can assume that additional changes occur also in the molecular composition of cells,

Table 5.1. Total protein nitrogen, non-protein nitrogen, and proportion of non-protein nitrogen in 'high' and 'low' genotypes.

Total protein nitrogen was determined by a Kjeldahl automatic analyser (Williams and Twine, 1967). Non-protein nitrogen was assayed according to Freney et al. (1977). 500 mg ground lucerne was boiled in 10 ml of 70% ethanol for 10 min. The suspension was filtered through a Whatman No. 42 filter and washed several times with boiling 70% ethanol until 100 ml was collected. The ethanol was evaporated under vacuum pressure. The filtrate was assayed for non-protein nitrogen by Kjeldahl automatic analyser (Williams and Twine, 1967).

Table 5.1. Total protein nitrogen, non-protein nitrogen, and proportion of non-protein nitrogen in 'high' and 'low' genotypes.

'high' protein genotype	Total nitrogen (mg/100 mg)	Non-protein nitrogen (mg/100 mg)	Proportion of non-protein nitrogen (% of total nitrogen)
1	3.67	0.75	20.4
2	3.29	0.60	18.2
3	2.86	0.75	26.2
4	3.92	0.84	21.4
Mean	3.44	0.74	21.55
'low' protein genotype			
1	2.25	0.51	22.7
2	2.46	0.57	23.2
3	2.29	0.57	24.9
4	2.56	0.51	19.9
Mean	2.39	0.54	22.68

particularly in respect of the ratio of RuBPC-ase and "cytoplasmic" proteins. Isolated leaf proteins were fractionated by Sepharose 6B column chromatography and the ratios of the isolated RuBPC-ase and "cytoplasmic" protein of the 'high and 'low' protein genotypes were compared. Four genotypes of the High and Low selections from the 3rd selection cycle (Table 5.2) were included in this experiment. There was considerable variation in the ratio of the two proteins within the 'high and 'low protein genotypes. No statistically significant difference was found between the mean values (5.58 and 4.74) of the two selections. It can be concluded that High and Low protein selection does not change the ratio of RuBPC-ase and "cytoplasmic" proteins in the cells of M. sativa. It seems that there is a mechanism regulating protein synthesis, and this balance in proportion of proteins may well have some biological significance.

5.2.4. Effect of Selection on Cell and Chloroplast Morphology.

Previous experiments showed that neither the ratio of RuBPC-ase to "cytoplasmic" proteins (Chapter 5.2.3.), nor the percentage of non-protein nitrogen (Chapter 5.2.2.) change during three selection cycles. Follow-up experiments examined the relationship between protein content of cells and cellular morphology. Fig. 5.2 to Fig. 5.5 show the cell preparations of two 'low' protein genotypes [(14) 1 and (15) 4, photo 1 and 3] and two 'high' protein [(6) 2 and (1) 44, photo 2 and 4] plant types. Genotypes (14) 1, (15) 4, and (6) 2 (photo 1, 3,2) have about the same cell area, but the cell area of genotype (1) 44 (photo 4) is nearly twice that of the other genotypes. The average plastid numbers are the same in cells, but the plastid areas of genotypes (1) 44 (photo 4) and (6) 2 (photo 2) are more than twice those of (14) 1 (photo 1) and (15) 4 (photo 3) samples.

Table 5.2. Ratios of RuBPC-ase to 'cytoplasmic' protein fractions of 'high' and 'low' protein genotypes after three selection cycles.

"High' and 'low' protein genotypes were derived from a selection experiment (Chapter 5.2.1.). RuBPC-ase and 'cytoplasmic' proteins were separated by Sepharose 6B column chromatography (Chapter 2.4.2). Protein samples were dialyzed against distilled water and freeze dried. Weights of freeze dried samples were used to calculate the ratio of RuBPC-ase to 'cytoplasmic' proteins.

Table 5.2. Ratios of RuBPC-ase to 'cytoplasmic' protein fractions of 'high' and 'low' protein genotypes selected after three selection cycles.

'high' Genotypes	RuBPC-ase / Cytoplasmic protein	'low' Genotypes	RuBPC-ase / Cytoplasmic protein
3 17	4.00	14 6	5.86
1 15	5.17	15 2	3.37
6 2	5.88	14 22	4.50
1 44	7.27	14 15	5.24
Mean	5.58	Mean	4.74

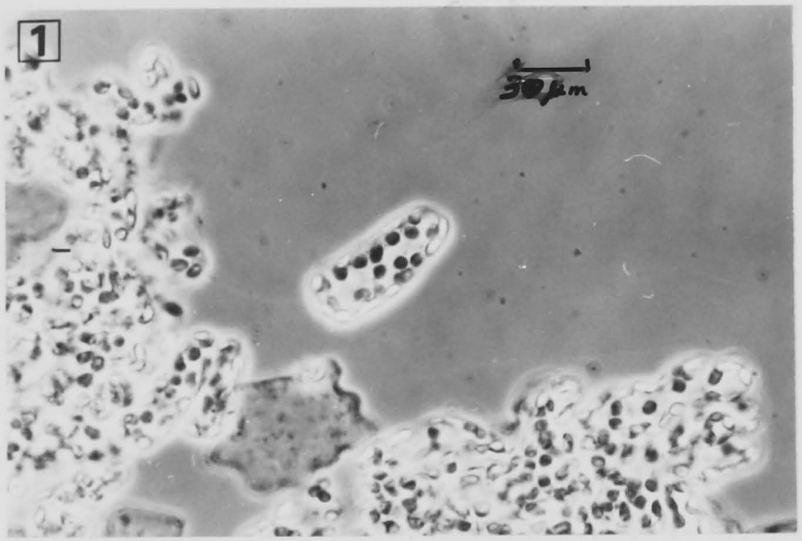
Fig. 5.2. Cells of 'low' protein genotype: (14) 1 (photo 1).

Fig. 5.3. Cells of 'high protein genotype: (6) 2 (photo 2).

Fig. 5.4. Cells of 'low' protein genotype: (15) 4 (photo 3).

Fig. 5.5. Cells of 'high" protein genotype: (1) 44 (photo 4).

The same methods were used for all the above Figs. Four 2 mm discs were cut from each of three leaflets. The discs were fixed in 3% gluteraldehyde in 0.05 M phosphate buffer, pH 7.3, and stored at 4°C. Half of the number of discs of each plant sample were transferred into 0.5 M EDTA, pH 9.0, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before covering with a coverslip. These preparations were used to determine chloroplast number, plastid diameter, and cell area. A Zeiss microscope (x 40 phase plan x 2 optivar, x 10 eyepieces) was used for the observations.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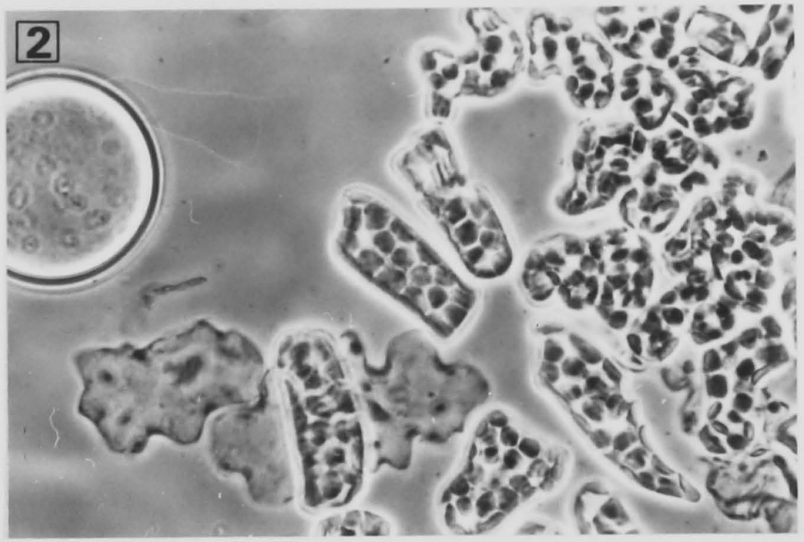
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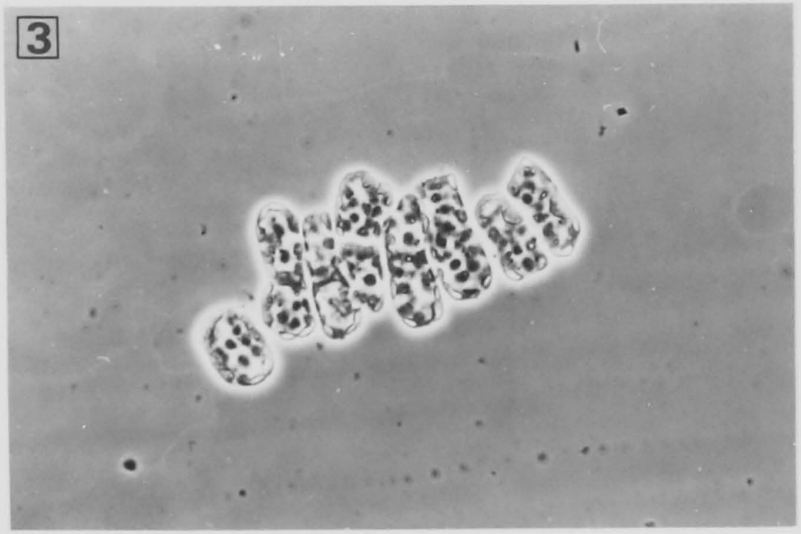
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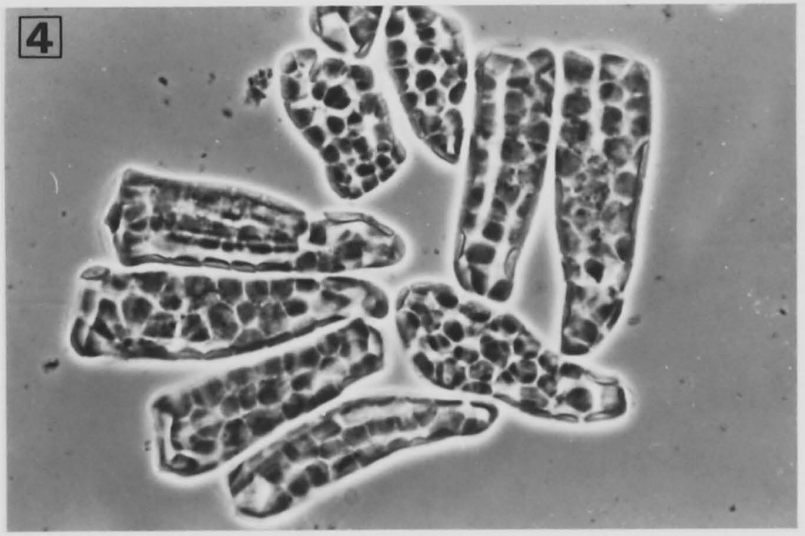
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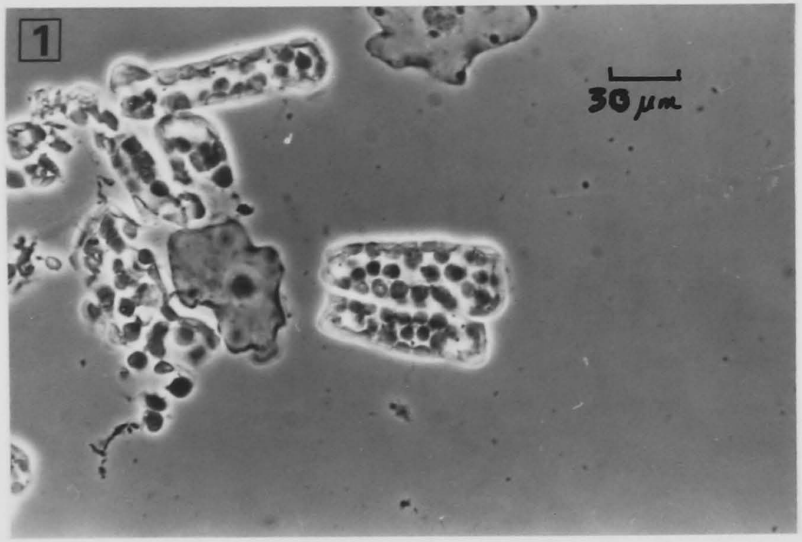
Fig. 5.6. Cells of a 'low protein genotype: 14.9 (photo 1).

Fig. 5.7. Cells of a 'high' protein genotype: 4.7 (photo 2).

Fig. 5.8. Cells of a 'low' protein genotype: 14.29 (photo 3).

Fig. 5.9. Cells of a 'high' protein genotype: 3.17 (photo 4).

The same methods were used for all 4 figures. For details of these methods, see legend to Figs. 5.2-5.5.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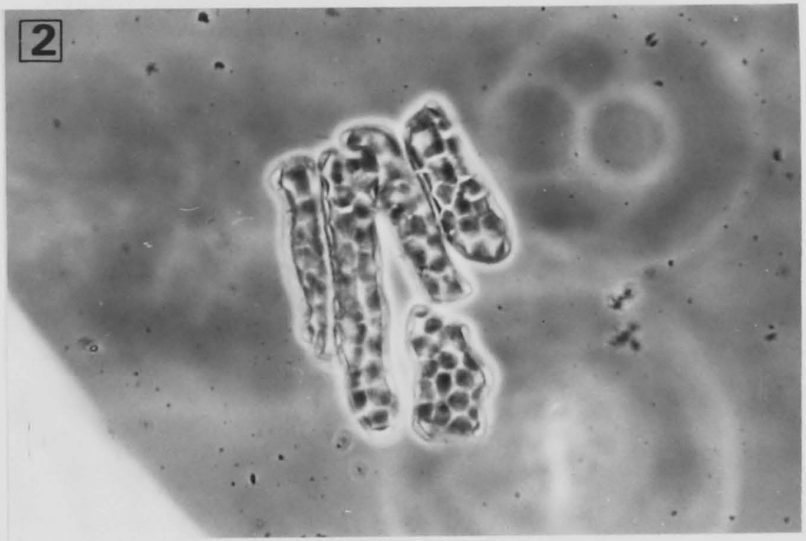
30 μm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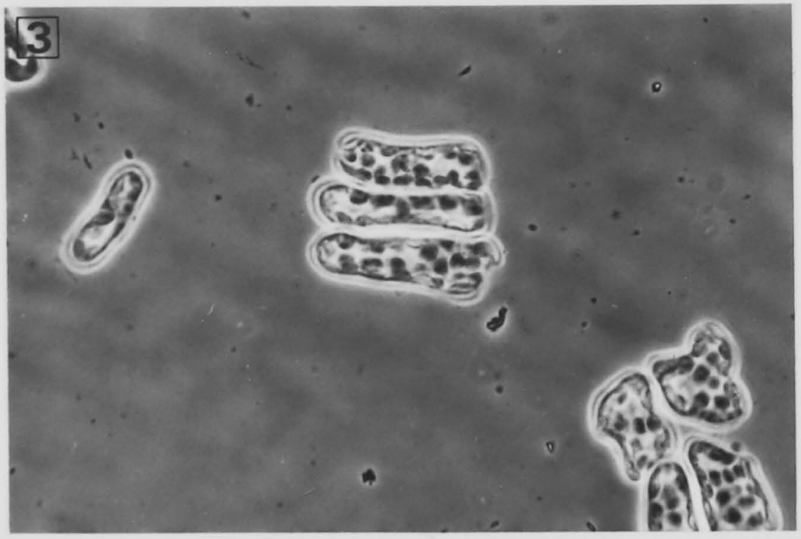
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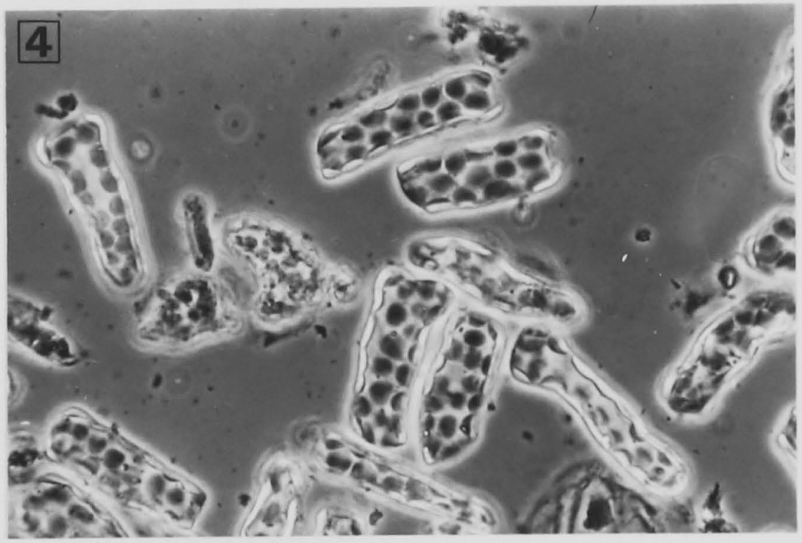
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Other cell preparations of (14) 9, (4) 7, (14) 29 and (3) 17 genotypes (Fig. 5.6-5.9, photos 1-4) show similar average cell areas. Cell number per 2mm disc, and average plastid number per cell were the same, but the plastid area of the two 'high' protein genotypes (photos 2 and 4) was twice the plastid area of the two 'low' protein genotypes (photo 1, 3).

It can be concluded that 'high' protein content of cells is correlated with large plastid area (size).

Measurements of protein content, plastid number per cell, plastid area (μm^2), number of cells (2 mm disc) and cell area ($mm^2 \times 10^{-4}$) of four 'high' and four 'low' protein genotypes are presented in Table 5.3. The mean percent protein of the 'high' genotypes was 29% and that of the 'low' genotypes was 17%. No significant statistical difference was found between the mean measurements of plastid number per cell, cell number per 2 mm disc, and cell area ($mm^2 \times 10^{-4}$). The mean plastid area of the 'high' genotypes was 31.00 μm^2 and that of the 'low' genotypes was 12.61 μm^2, with the difference being statistically highly significant ($P < 0.001$).

Large plastids from cells of a 'high' protein genotype are shown in Fig. 5.10. The plastids occupy the entire space of the cells. In contrast the cells of 'low' protein genotypes (Fig. 5.11). have smaller plastids and there are considerable gaps between the plastids and the cell walls. These results suggest a correlation between protein synthesis and plastid area.

Further investigations concerned the effect of developmental stage on the plastid and cell morphology of 'high' and 'low' protein genotypes. Fig. 5.12 (photo 1) shows cells of young (unfolded) leaves of a 'low' protein genotype (14.9). There are fewer and smaller plastids and the cell area is also smaller, as compared with

Table 5.3. Relationship between 'high' and 'low' leaf protein genotypes and cell or chloroplast characteristics.

Four 2 mm discs were cut from each of the three leaflets, midway between the midrib and leaf margin. The discs were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.3 and stored at 4°C. Six discs of each plant sample were transferred to 3 ml of 5% $K_2Cr_2O_7$ in 1 N HCl, 60°C, for 2.5 to 3 h, until they could be separated into single cells by gently pipetting up and down in the test tube. The remaining six discs were transferred to 0.05 M EDTA, pH 9, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before placing a coverslip on top. This preparation was used to determine chloroplast number, plastid diameter and cell area.

The details of the determination of plastid numbers per cell (Chapter 2.10.4), plastid area (Chapter 2.10.5), cell number per disc (Chapter 2.10.6) and cell area (Chapter 2.10.7) were presented in Materials and Methods.

Table 5.3. Relationship between 'high' and 'low' leaf protein content of genotypes and chloroplast characteristics.

Genotype	Protein (%)	Plastid no. per cell	Plastid area (μm^2)	Cell no. per 2 mm disc.	Cell area ($mm^2 \times 10^{-4}$)
'high'					
1 44	29	29.8	34.38	45,203	12.27
3 17	28	29.8	26.65	54,817	7,75
4 7	27	29.5	32.05	57,630	6.80
6 2	32	28.3	30.93	62,761	6.78
Mean	29	29.35	31.00	55,102	8.33
'low'					
14 1	12	29.7	12.69	55,598	6.47
14 9	22	29.5	13.80	48,594	6.21
14 29	17	28.8	12.84	57,859	6.49
15 4	18	27.8	11.13	51,692	5.87
Mean	17	28.95	12.61	53,435	6.26

Fig. 5.10. Cells of a 'high' protein genotype (1.44).

Fig. 5.11. Cells of a 'low' protein genotype (15.4).

The same methods were used for both figures. For details of these methods, see legend to Fig. 5.2-5.5.


5 μm

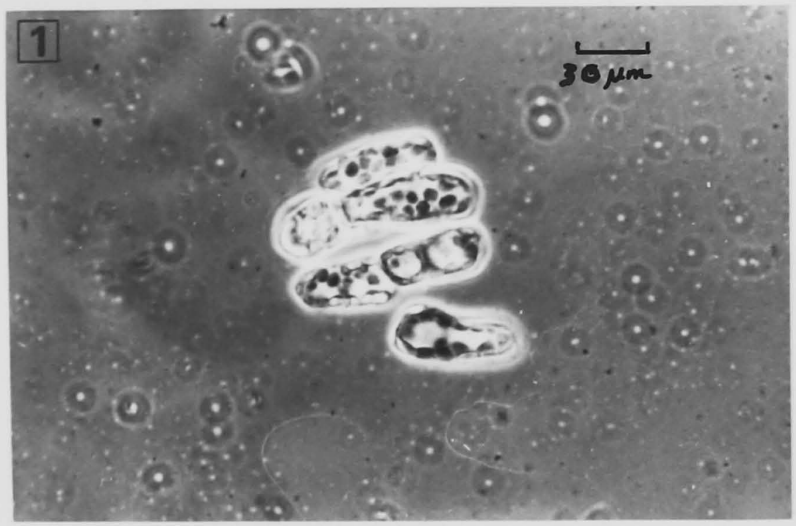
Fig. 5.12. Cells of young leaves of a 'low' (14.9) protein genotype (photo 1).

Fig. 5.13. Cells of young leaves of a 'high' (6.2) protein genotype (photo 2).

Fig. 5.14. Cells of old leaves of a 'low' (14.9) protein genotype (photo 3).

Fig. 5.15. Cells of old leaves of a 'high (6.2) protein genotype (photo 4).

The same methods were used for all 4 figures. For details of these methods see legend to Figs. 5.2-5.5.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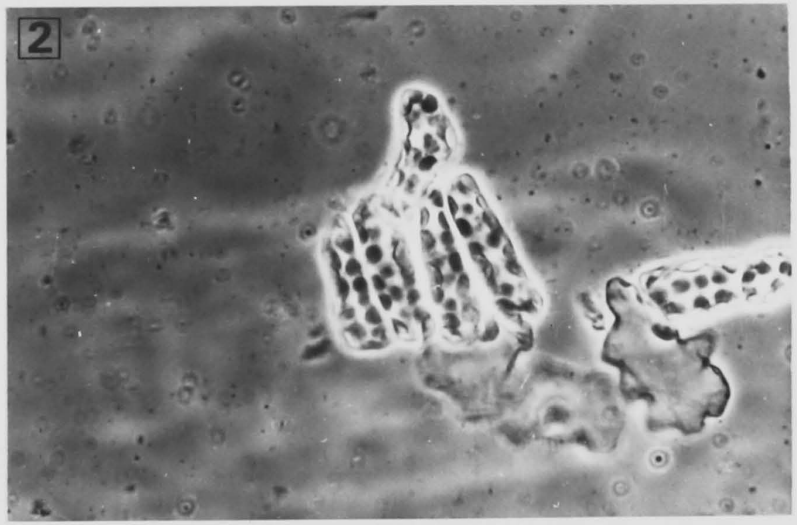
30 µm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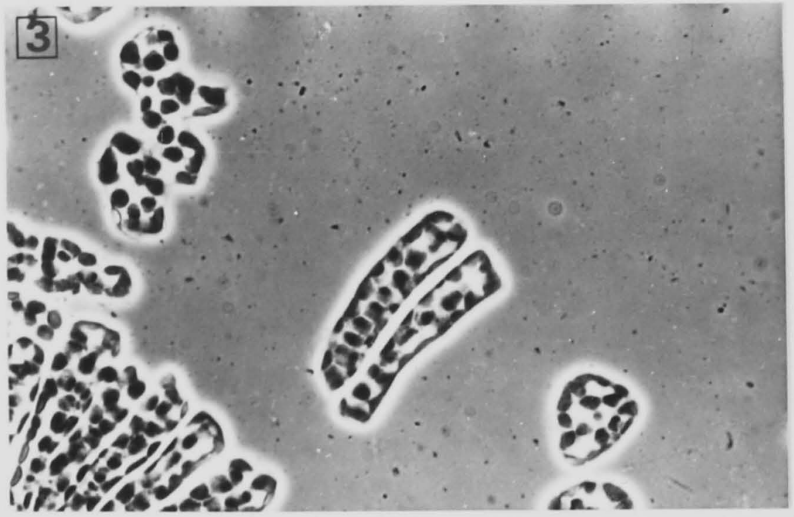
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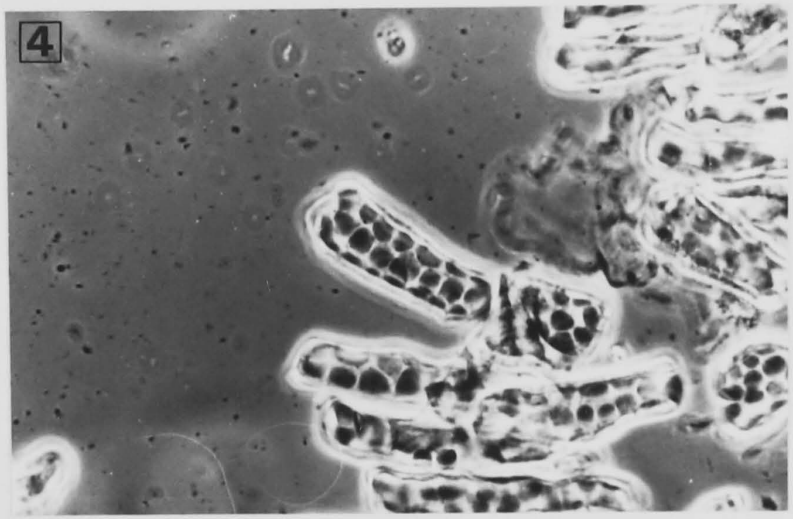
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the same parameters in older leaves (Fig. 5.14, photo 3) of the same 'low' protein genotype. Similarly, the plastid number, plastid area and cell area of young (unfolded) leaves of a 'high protein genotype (6.2) are less (Fig. 5.13, photo 2) than in the case of older leaves of the same genotype (Fig. 5.15, photo 4). When cells of young and old leaves of the 'high protein genotype (photo 2 and 4) are compared to the cells of young and old leaves of the 'low' protein genotype (photo 1 and 3), it is apparent that the plastid area is greater in both young and old leaves of the 'high' genotype. It can be concluded that differences in plastid area of 'high' and 'low' protein genotypes are expressed at both early and late developmental stages.

The effect of leaf developmental stage ("young" versus "old" leaves) on plastid numbers and cell area is shown by the measurements given in Table 5.4. Plastid number increased with leaf age from 17.7 to 26.7 and from 21.7 to 26.7 per cell in 'low' (14.9) and 'high' (6.2) genotypes, respectively. Plastid area also increased from 11.76 to 15.55 and from 17.46 to 30.40 μm^2, respectively, for the two genotypes. Cell area ($mm^2 \times 10^{-4}$) was also greater in older leaves of both genotypes. Plastid area of both young and old leaves was greater in the 'high' (6.2) protein genotype than in the 'low' (14.9) genotype. These numerical analyses support the microscopical results and show that the greater plastid area of the 'high' genotype is expressed in all developmental stages.

The relationship betwen plastid size and protein content was also examined in segregating populations (Fig. 5.1). Four genotypes were selected. Two genotypes were derived from 'high' (♀) x 'low' (♂) protein (H x L) crosses; one of them (H x L, 6) had 32.1% protein and the other (H x L, 19) contained 12.0% protein. Two selections were also obtained from reciprocal 'low (♀) x 'high' (♂) protein (L x

Table 5.4. Effect of developmental stage on chloroplast morphology and cell area.

Four 2 mm disc were cut from each of the three leaflets midway between the midrib and leaf margin. The discs were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer pH 7.3 and stored at 4°C. Six discs of each plant sample were transferred to 3 ml of 5% $K_2Cr_2O_7$ in 1 N HCl, 60°C, for 2.5 to 3 h, until they could be separated into single cells by gently pipetting up and down in the test tube. The remaining six discs were transferred to 0.05 M EDTA, pH 9, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before placing a coverslip on top. This preparation was used to determine chloroplast number, plastid diameter and cell area.

The details of determination of plastid numbers per cells (Chapter 2.10.4), plastid area (Chapter 2.10.5) and cell area (Chapter 2.10.7) were presented in Materials and Methods.

Table 5.4. Effect of developmental stage on chloroplast morphology and cell area.

Genotype	Develop. stage	Plastid no. per cell	Plastid area (μm^2)	Cell area ($mm^2 \times 10^{-4}$)
14.9	Young	17.7	11.76	3.65
	Old	26.7	15.55	6.73
6.2	Young	21.7	17.46	5.66
	Old	26.7	30.40	6.72

Table 5.5. Percent plant protein, mean plastid number per cell, and mean plastid area for progenies of H x L and L x H crosses.

Four 2 mm disc were cut from each of the three leaflets midway between the midrib and leaf margin. The discs were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.3 and stored at 4°C. Six discs of each plant sample were transferred to 3 ml of 5% $K_2Cr_2O_7$ in 1 N HCl, 60°C, for 2.5 to 3 h, until they could be separated into single cells by gently pipetting up and down in the test tube. The remaining six discs were transferred to 0.05 M EDTA, pH 9, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before placing a coverslip on top. This preparation was used to determine chloroplast number, plastid diameter and cell area.

The details of determination of plastid numbers per cells (Chapter 2.10.4) and plastid area (Chapter 2.10.5), were presented in Materials and Methods.

Table 5.5. Percent plant protein, mean plastid number per cell, and mean plastid area for progenies of H x L and L x H crosses.

Genotype	% protein	Plastid no. per cell	Plastid area (μm^2)
L x H, 16	21.5	30.0	15.14
L x H, 18	17.0	25.2	5.40
H x L, 6	32.1	29.6	13.46
H x L, 19	12.0	22.9	4.96

H) crosses; one (L xH, 16) contained 21.5% protein and the other (L x H, 18) had 17.0% protein (Table 5.5). Cells of the two 'high' protein genotypes (L x H, 16 and H x L, 6) are shown in Fig. 5.16 (photo 1) and Fig. 5.18 (photo 3). Cells of these two 'high' protein genotypes had much greater plastid area than those of 'low' protein genotypes (Fig. 5.17. L x H, 18, photo 2; Fig. 5.19, H x L, 19, photo 4). These findings indicate that the correlation between plastid area and protein content also applies in segregating populations.

The mean values for plastid number per cell and for plastid area (μm^2) of 'high' and 'low' protein genotypes are presented in Table 5.5 Plastid numbers were 25.2 and 22.9 for the 'low' protein genotypes (L x H, 18; H x L, 19); those for the 'high' protein genotypes (L x H, 16; H x L, 6) were 30.0 and 29.6. The differences between plastid areas of the two types of genotypes were even greater. Areas for the two 'low' protein genotypes, were 5.40 and 4.96 μm^2; those for the two 'high' protein genotypes were 15.14 and 13.46 μm^2.

5.2.5. Chloroplast Morphology in HP and DP Genotypes of Medicago.

The same parameters as above were also studied in HP and DP (DuPuits) cultivars of Medicago. These two cultivars differed in protein content (Table 5.6). The HP cultivars had a lower mean plastid number (23.8) and smaller plastid area (5.56 μm^2) than the DP cultivar, which had a plastid number of 29.6 and an area of 16.68 μm^2. It can be concluded that the correlation between percent protein and plastid area applies to established cultivars as well.

No differences were observed between the growth of 'high' protein plant (Fig. 5.20, 2) and 'low' protein plants (Fig. 5.20. 1). However, leaf colour (indication of chlorophyll content) is very

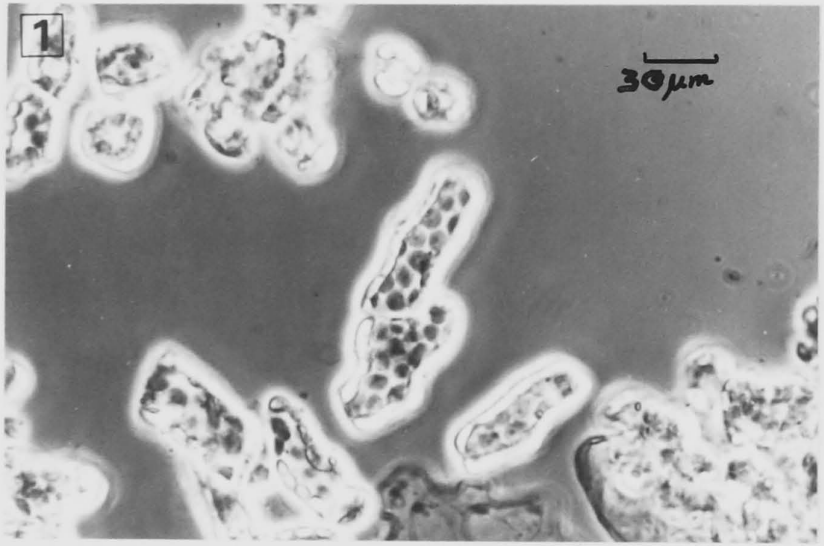
Fig. 5.16. Cells of a 'high' protein genotype (L x H, 16) derived from 'low' by 'high crosses (photo 1).

Fig. 5.17. Cells of a 'low' protein genotype (L x H, 18) derived from 'low' by 'high' crosses (photo 2).

Fig. 5.18. Cells of a 'high' protein genotype (H x L, 6) derived from 'high' by 'low' crosses (photo 3).

Fig. 5.19. Cells of a 'high' protein genotype (H x L, 19) derived from 'high' by 'low' crosses (photo 4).

The same methods were used for all 4 figures. For details of these methods, see legend to Figs. 5.2-5.5.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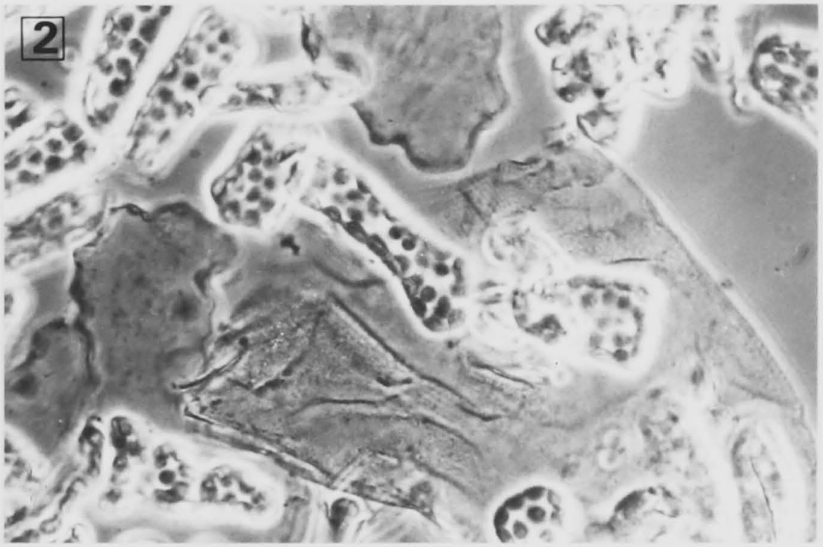
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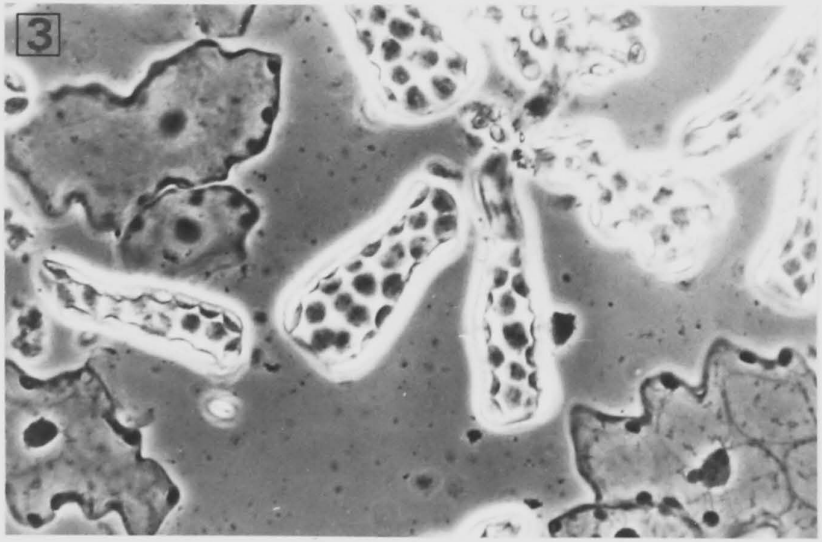
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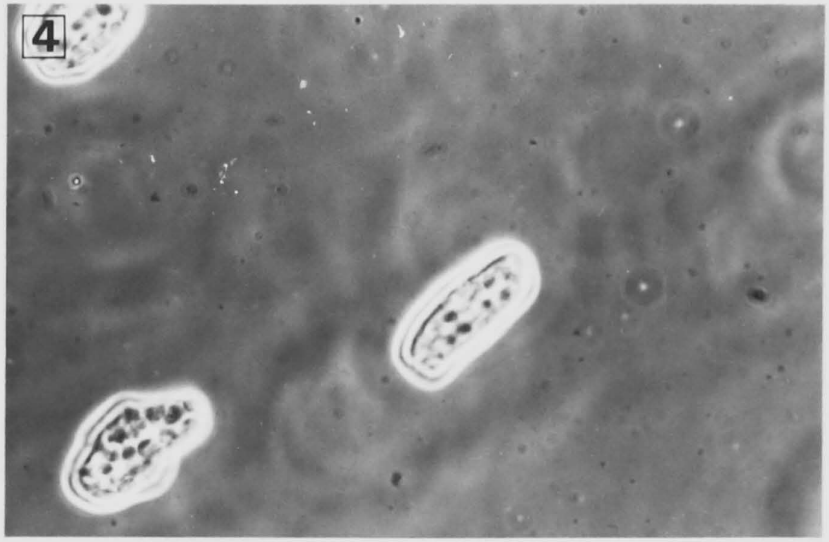
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Table 5.6. Percent protein, plastid number, and plastid area for HP and DP genotypes.

Four 2 mm discs were cut from each of the three leaflets, midway between the midrib and leaf margin. The discs were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.3, and stored at 4°C. Six discs of each plant sample were transferred to 3 ml of 5% $K_2Cr_2O_7$ in 1 N HCl, 60°C, for 2.5 to 3 h, until they could be separated into single cells by gently pipetting up and down in the test tube. The remaining six discs were transferred to 0.05 M EDTA, pH 9, at 60°C for 2 h. Each disc was then macerated with a pair of jeweller's forceps on a glass slide and the pool of cells mixed with 50% glycerol before placing a coverslip on top. This preparation was used to determine chloroplast number, plastid diameter, and cell area.

The details of determination of plastid numbers per cells (Chapter 2.10.4) and plastid area (Chapter 2.10.5) were presented in Materials and Methods.

Table 5.6. Percent protein, plastid number, and plastid area for HP and DP genotypes.

Genotype	% Protein	Plastid no. per cell	Plastid area (μm^2)
HP	17	23.8	5.56
DP	22	29.6	16.68

Fig. 5.20. Low (1) and high (2) protein plants.

Fig. 5.21. Leaves of low (1) and high (2) protein plants.

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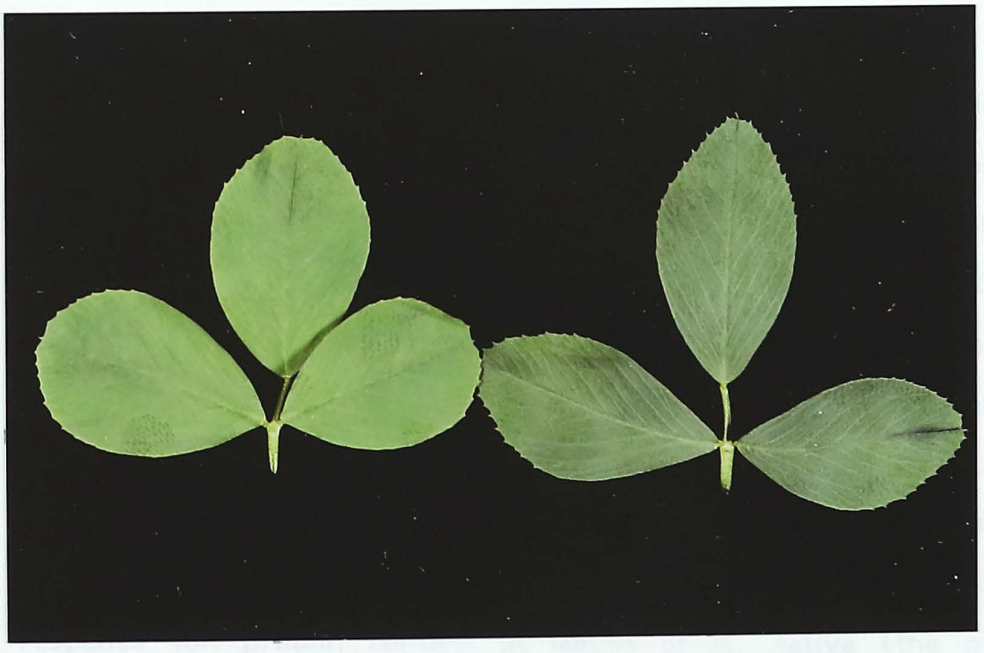

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different; the colour of the 'high' protein genotype (Fig. 5.20, 2) is much darker than that of the 'low' protein genotype (Fig. 5.21, 1).

5.3 DISCUSSION

The results of this chapter established several significant findings concerning selection for protein content in M. sativa. Protein content of leaves increased during three generations of selection and change in RuBPC-ase and 'cytoplasmic' proteins also occurred, but the ratio of "High" and "Low" selections remained the same. Chloroplast area was correlated with leaf protein content. This correlation was also confirmed in hybrid generations and in established cultivars.

The mean protein content of plants of M. sativa increased by 9.5% during three hybrid generations which can be explained largely by genetical but may contain some environmental factors. As far as I am aware, no selection experiments have previously been carried out for high leaf protein content in any plant species. Selection experiments for high and low protein content of the grain of corn (Zea mays L.) were conducted during seventy generations by Dudley et al. (1974). The protein content of the basic population was 10.9% and selection for high protein produced grain with 26.6% and selection for low protein produced grain with 4.4% after 70 generations. The increase in protein content was 0.7% and the decrease was 1.4% during the first three selection generations. The rate of selection response for protein content in leaves of M. sativa (the present study) was much greater than in the grains of Z. mays.

The amino acid composition of chloroplast and 'cytoplasmic' proteins in Medicago (Chapter 3.2.1. iv) showed a slight deficiency of methionine, in contrast to deficiencies of lysine and tryptophan as found in Z. mays (Alexander and Creech 1977).

Selection for high and low protein content over three generations was effective in Vicia faba. Seed weight was positively correlated with protein content. Protein content was positively correlated with arginine content (r = + 0.80), and negatively correlated with lysine (r = -0.92) and methionine (r = -0.54) content but was not correlated with cysteine (Sjödin, 1981).

Non-protein nitrogen as a percent of total nitrogen was the same in 'high' and 'low' protein genotypes of Medicago. In contrast to these results with leaf proteins, the proportion of free amino acids was higher in seeds of cultivars of barley which had a high protein content (Balaravi et al., 1976).

Considerable differences were found between the published heritability values of RuBPC-ase and 'cytoplasmic' proteins (see Chapter 5.1) and the results of my experiments. These differences can be explained by the different methods of isolation and determination of leaf proteins. The high realized heritability value of M. sativa in the present experiment was also supported by the great increase in protein content of leaves.

RuBPC-ase content of extreme genotypes (14.1 = 11.5% and 6.2 = 32%) was the result of intense selection (Chapter 5.2.1.) and it was achieved without any increase in DNA content. The ratios of RuBPC-ase to DNA were very similar across ploidy levels, suggesting that RuBPC-ase increases proportionately with the amount of DNA per cell (Meyers et al., 1982a). It can therefore be concluded that increase in RuBPC-ase content of cells can be achieved by accumulating high-yielding genes or by increasing the DNA content.

The correlation between large plastid area and high RuBPC-ase synthesis (estimated from high protein content) can be tentatively explained by the greater amount of LSU synthesis, which appears to be favoured by a larger plastid volume.

Variation of RuBPC-ase activity in different plant species was attributed to several environmental factors in addition to genetic influence.

Diurnal variation of light can influence the activity of RuBPC-ase in peanut leaves (Ku et al., 1982). The activity of peanut leaf RuBPC-ase in crude extracts increased sharply in plants harvested during the first hour of light, reached a plateau after four hours in light, and remained high throughout the rest of the photoperiod. When leaves were exposed to darkness the enzyme activity remained high for an hour but decreased drastically thereafter. Photosynthetic metabolites such as 6-phosphogluconate, fructose-1,6-bisphosphate, and sedoheptulose-1,7-bisphosphate, when added to the assay medium at 1 mM concentration, inhibited the enzyme. With purified RuBPC-ase, these metabolites act as competitive inhibitors of the enzyme. The concentrations of these metabolites in leaf tissues are 2-5 fold higher in the dark than in the light. RuBPC-ase activity in leaf extracts prepared during the light period decreased while that in the leaf extracts prepared during the dark period increased steadily with time of dialysis against buffer solution. These results suggest that light may regulate the diurnal activity of RuBPC-ase by affecting the levels of these photosynthetic metabolites.

Seasonal variation of various enzyme activities was demonstrated by Küppers and Weidner, (1980). The pattern of seasonal variation of enzyme levels in the brown alga Laminaria hyperborea (Gunn.) Fosl. was investigated for the following enzymes: RuBPC-ase, phosphoenol-pyruvate-carboxykinase, glyceraldehyde-3-phosphate-dehydrogenase, malate-dehydrogenase, L-aspartate-2-oxoglutarate-amino-transferase, and mannitol-1-phosphate-dehydrogenase. The first four enzymes

exhibited a circannual periodicity characterized by a pronounced 'spring-maximum' of enzyme activity in April and May. As a consequence, the phylloid maintained high metabolic rates from early spring on, although water temperature had then risen only slightly above the annual minimum. The seasonal pattern correlated well with circannual fluctuations of the nitrogen content of the sea and with variation of the internal nitrogen and nitrate content of the alga. This coincidence may indicate that the nitrogen level plays an important role in the regulation of enzyme activities, and hence the metabolic capacities of L. hyperborea.

Another variable affecting RuBPC-ase was found to be associated with the developmental stage of pods in Pisum sativum L. (Price and Hedley 1980). The activities of phosphoenol pyruvate carboxylase and RuBPC-ase were determined for the developing pod wall cells of six genotypes of Pisum sativum. Whilst both enzymes were detected in plants of all genotypes, the levels of activity varied considerably with pod type and age.

Finally, it was found that level of nutrient affected the activity of RuBPC-ase and fructose bisphosphatase in developing barley leaves (Fritsch, 1982). Leaves of plants grown at a low nutrient level had markedly depressed enzyme activity and protein content but had a higher carbohydrate concentration as compared to plants grown at a higher nutrient level. It was concluded that in developing leaves additional fertilizer directs carbon flow into protein and structural components rather than into carbohydrates.

The environmental and developmental factors discussed above appear to be important contributors to the activity of RuBPC-ase of plant species and, in addition to the genetic variability, should be seriously considered in any plant improvement program.

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CHAPTER 6.

EFFECT OF TEMPERATURE AND LIGHT INTENSITY ON THE SYNTHESIS OF LSU AND SSU IN HP AND MF GENOTYPES OF MEDICAGO

6.1. INTRODUCTION

Evidence for genetic control of synthesis of RuBPC-ase and "Cytoplasmic" proteins in M. sativa was presented in the preceding chapter. It was concluded that increased level of RuBPC-ase is under nuclear DNA control. The present chapter examines the effect of environmental factors on the synthesis of LSU and SSU.

The synthesis of SSU in the absence of LSU of RuBPC-ase in 70S ribosome-deficient rye (Secale cereale L.) was studied by Feierabend and Wildner (1978). Immunological tests with antisera monospecific to RuBPC-ase and to its LSU or SSU indicated the presence of a protein with antigenic properties of SSU and the absence of the LSU in the leaves of young rye plants which also showed a high temperature-induced (32°C) deficiency of 70S plastid ribosomes. The SSU-like protein was isolated from crude extracts of plastids from ribosome-deficient (32°C-grown) leaf tissue by the use of chromatography columns with immobilized antibody. The main polypeptide retained by the immobilized antibody had the same mobility after electrophoresis on SDS polyacrylamide gels as the SSU of RuBPC-ase and was also immunologically identical to the SSU. The SSU-like protein was present in the supernatant as well as in the membrane fraction of isolated 70S ribosome-deficient plastids. At very young stages of normal leaves grown at a permissive temperature (22°C), an excess of SSU which was observed was not integrated into

the complete RuBPC-ase molecule. It was concluded that synthesis of the SSU occurs on cytoplasmic ribosomes and was not strictly co-ordinated with the translation of the LSU in the chloroplast. During early leaf development, formation of LSU seems to be the rate limiting step in the synthesis of RuBPc-ase.

The effect of temperature changes on the activity of photosynthetic enzymes was investigated in leaves of maize (Z. mays L.) seedlings (Stamp, 1980). Seedlings of maize were first grown at 24/22°C (high) or 14/12°C (low) day/night regimes; the temperature was then changed from high to low or from low to high. After growth at "low" compared to "high" temperature the genetic variability was considerably higher in a number of enzyme characteristics. Generally the following differences were induced by growth at low as compared to high temperature : lower chlorophyll content; slightly higher carotenoid content; slightly higher PEP-carboxylase activity; lower RuBPC-ase activity; no change in NADP-malate dehydrogenase activity. Transition from high to low temperature had the following effects : slight decrease in the chlorophyll content for about two days; increase in the carotenoid content, rapid or only after some days; slight increase in PEP carboxylase activity; insignificant change in RuBPC-ase activity; no change in NADP-malic enzyme activity; sharp decrease in NADP-malate dehydrogenase activity; no severe retardation of shoot fresh weight accumulation. Transition from low to high temperature had the following effects : fast and steady increase in chlorophyll and carotenoid content; slight decrease of PEP-carboxylase activity; a fast and steady increase in RuBPC-ase activity; no change in NADP-malic enzyme activity; temporary decrease in NADP-malate dehydrogenase

activity; interruption of shoot fresh weight accumulation for one to two days. The implications of the above results for the photosynthetic efficiency of maize were that the RuBPC-ase and the NADP-malate dehydrogenase could possibly be limiting this process during periods of cool temperatures.

The other important environmental factor was light. Tobin and Suttie (1980) investigated the effect of light on the synthesis of RuBPC-ase in Lemna gibba L. G-3. L. gibba (an aquatic plant) was grown in light and thereafter placed in the dark, resulting in a changed pattern of protein synthesis. Although the amount of protein in the tissue and the over-all rate of incorporation of ^{35}S-methionine into protein did not significantly decline during four days of darkness, the rate of synthesis of three polypeptides declined dramatically. One of these polypeptides was the chlorophyll a/b-binding protein and the other two were the LSU and SSU of RuBPC-ase. The changed rates of synthesis of the two subunits were examined after transitions of plants from light to dark and dark to light. The in vivo synthesis of both subunits, while declining to a low level during four days of darkness, increased rapidly upon returning the plants to white light. In addition, the level of poly-(A) mRNA coding for the precursor polypeptide of the SSU of the enzyme also fell to a low level in the dark and increased rapidly in response to white light. The increase in translatable mRNA for the SSU was rapid enough to account for a major part of the increased synthesis of this subunit.

The aim of the present investigation was to examine the effect of normal (25/19°C) and high (35/30°C) temperature on the synthesis of total leaf proteins as well as LSU and SSU synthesis of two

geographically and genetically distinct genotypes (HP and MF) of Medicago. In addition, I investigated the effect of reduced daylight on total leaf protein, subunit synthesis, and genotype x low light intensity interaction in Medicago.

6.2. RESULTS

6.2.1. Determination of the Relative Amount of RuBPC-ase and 'Cytoplasmic' Proteins in Tissue Extracts

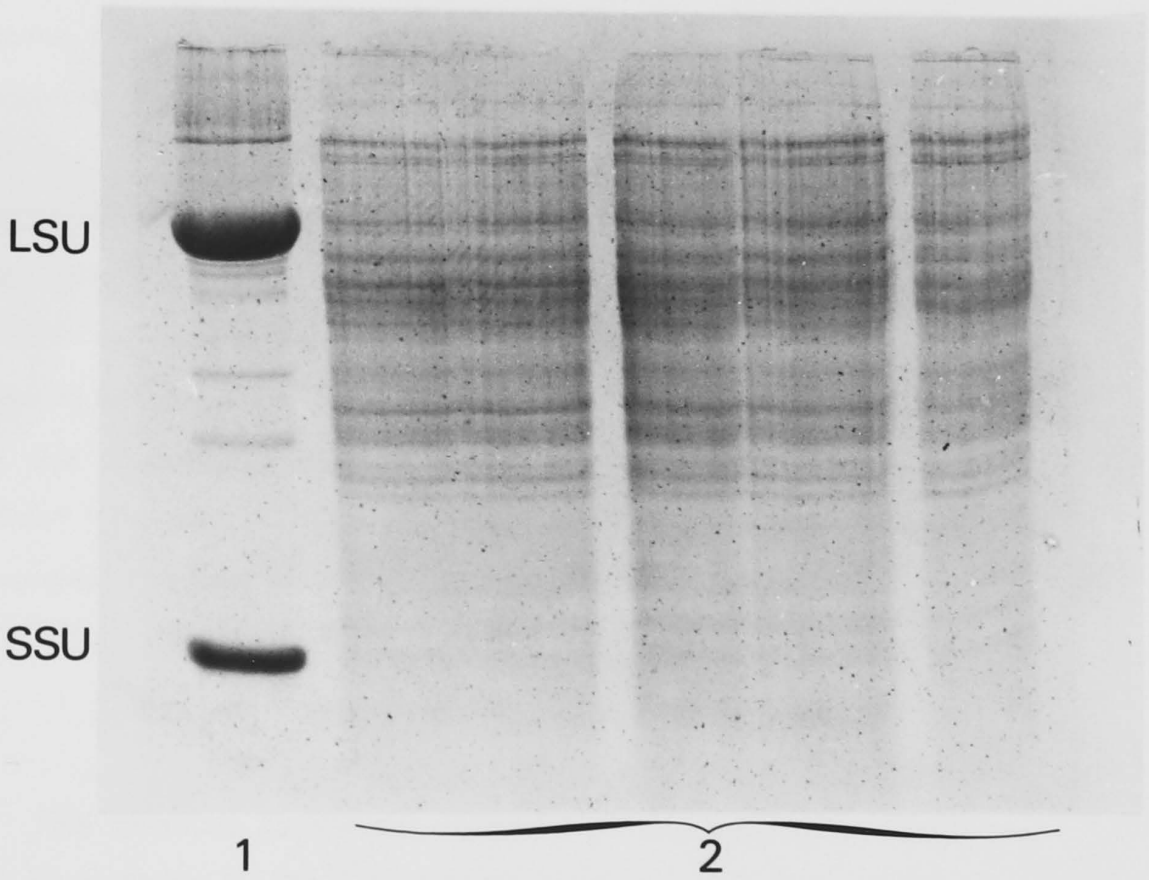
An important component in this work was the choice of an efficient method for determining LSU and SSU polypeptide content of cells. There are several methods available for isolation of total LSU and SSU subunits of RuBPC-ase from plant cells. I chose SDS gel electrophoresis as the most reliable and simple method for LSU and SSU analysis. The primary aim of this experiment was to determine the total amount of LSU and SSU polypeptides in chloroplast and 'cytoplasmic' proteins. Leaf extract of HR genotype was applied to a Sepharose 6B column and the peak fractions of RuBPC-ase and 'cytoplasmic' proteins were isolated. Preparations containing 50 µg each of RuBPC-ase or 'cytoplasmic' protein, SDS, and 2-mercaptoethanol were incubated and applied to a 13% SDS Davis gel. Electrophoresis was performed for 2 h. Results are presented in Fig. 6.1. Separation of polypeptides of RuBPC-ase is shown in lane 1 (Fig. 6.1). The LSU (53 kd) and SSU (14 kd) bands are the major polypeptides, although a number of minor bands are also visible. 'Cytoplasmic' proteins, applied to several slots of the Davis gel (Fig. 6.1, lane 2), produced numerous small bands. No band at all is apparent at 14 kd and a weak double band is apparent at 53 kd. It can be concluded from this gel analysis that the LSU and SSU polypeptides were found predominantly in intact RuBPC-ase.

Fig. 6.1. SDS gel electrophoretograms of RuBPC-ase and 'cytoplasmic' proteins.

Isolation of RuBPC-ase and 'cytoplasmic' proteins was performed by Sepharose 6B column chromatography (Chapter 2.4.2). The RuBPC-ase and 'cytoplasmic' protein peak fractions were collected. 50 µg of each RuBPC-ase and 'cytoplasmic' protein sample was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, then loaded on a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA, and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx 120 V at room temperature. The gels were stained in Coomassie Blue solution for 4 h (Chapter 2.8.7). Destaining was done with a 6:1:13 mixture of ethanol, glacial acetic acid, and water.

Lane 1: RuBPC-ase of the HR genotype.

Lane 2: 'cytoplasmic' proteins of the HR genotype.


LSU
SSU
1
2

The above gel was also used to compare scanning profiles of RuBPC-ase and 'cytoplasmic' proteins, using a Gilford spectrophotometer 240. Fig. 6.2 shows the profile of RuBPC-ase (upper scan) and of 'cytoplasmic' proteins (lower scan). The scan of RuBPC-ase shows the SSU and LSU polypeptide peaks and some minor peaks close to the LSU peak. The scan of the 'cytoplasmic' protein shows that there are many minor peaks at 2 to 6 cm from the origin of the gel. There is a very small peak at 6.5 cm from the origin, which corresponds to the SSU polypeptide region of the RuBPC-ase, and there are two small peaks between 2 to 2.5 cm from the origin which correspond to the LSU region of RuBPC-ase. This small amount of 'cytoplasmic' protein in the LSU region is not more than 8% of the total LSU polypeptide of RuBPC-ase and is considered to be "free" LSU polypeptide. The conclusion was that the predominant proteins of regions of SSU and LSU were the subunits of RuBPC-ase.

6.2.2. Variation of the Ratio of LSU to SSU

6.2.2. (i) Variation of the Ratio of LSU to SSU for RuBPC-ase of HR, HP and MF Genotypes

The previous experiment showed that the majority of 53 kd and 14 kd proteins in the cell extract were LSU and SSU polypeptides. The present experiment examines the possible variation in the ratio of LSU to SSU polypeptides of leaf extracts of established genotypes. Fig. 6.3 shows SDS gel electrophoretograms of RuBPC-ase of HR (lane 1), and total leaf proteins of HP (lane 2) and MF (lane 3) genotypes. RuBPC-ase of HR had LSU and SSU polypeptide bands and two minor bands. The extract of total leaf proteins of HP (lane 2) and MF (lane 3) showed numerous small bands apart from the two major LSU and SSU polypeptides. The ratio of LSU to SSU for RuBPC-ase of HP and MF genotypes did not differ significantly.

Fig. 6.2. (Upper scan) Densitometric scan profile of RuBPC-ase of HR genotype.

The gel was prepared as described for Fig. 6.1. The stained gels were cut vertically into slices (1 cm wide) and placed in a Gilford spectrophotometer-240. The wave length was set at 600 nm and the absorbance control at 1500.

Fig. 6.2. (Lower scan) Densitometric scan profile of 'cytoplasmic' proteins of HR genotype.

ABSORBANCE 600 nm

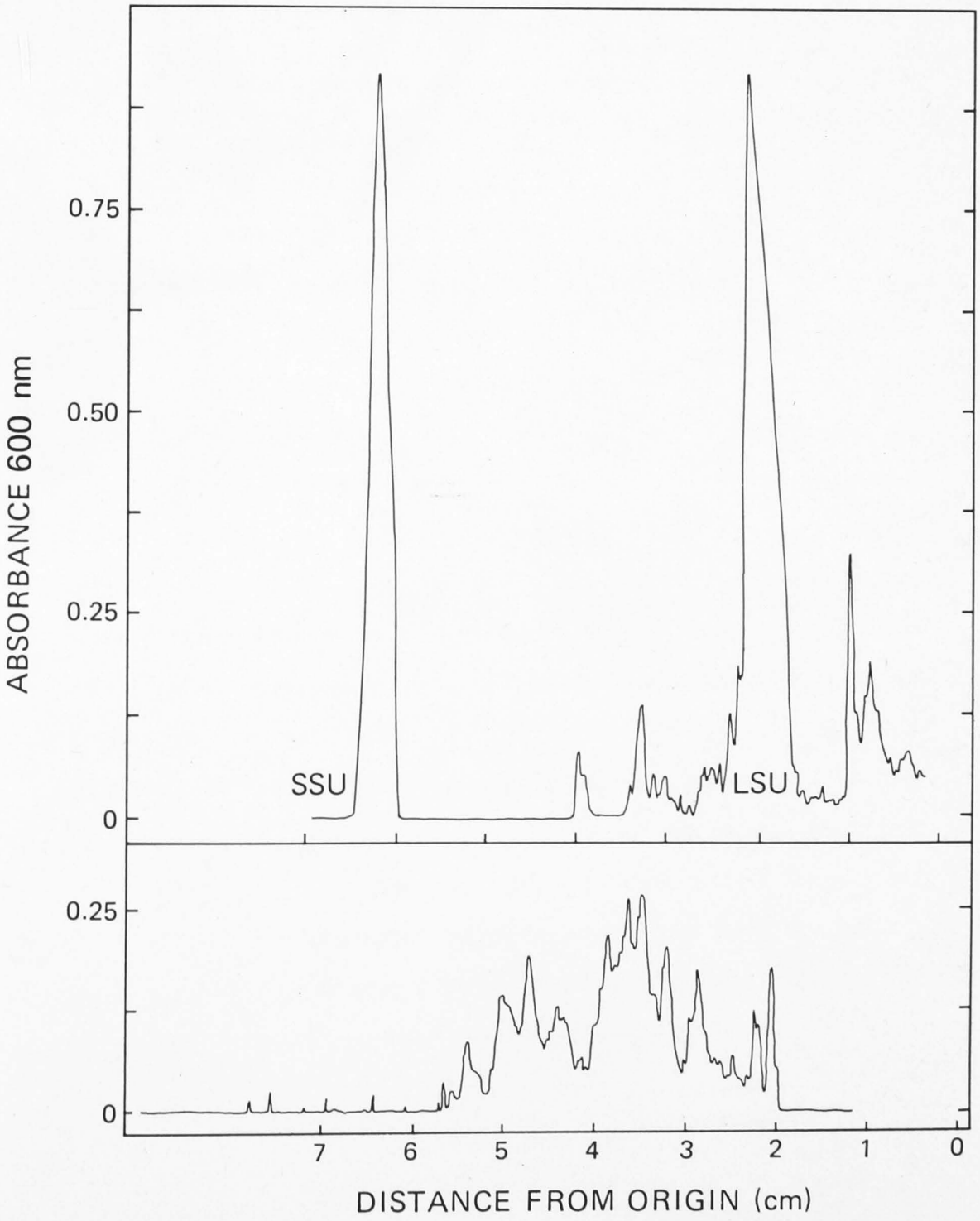

ABSORBANCE 600 nm
0.75
0.50
0.25
0
SSU
LSU
0.25
0
7
6
5
4
3
2
1
0
DISTANCE FROM ORIGIN (cm)

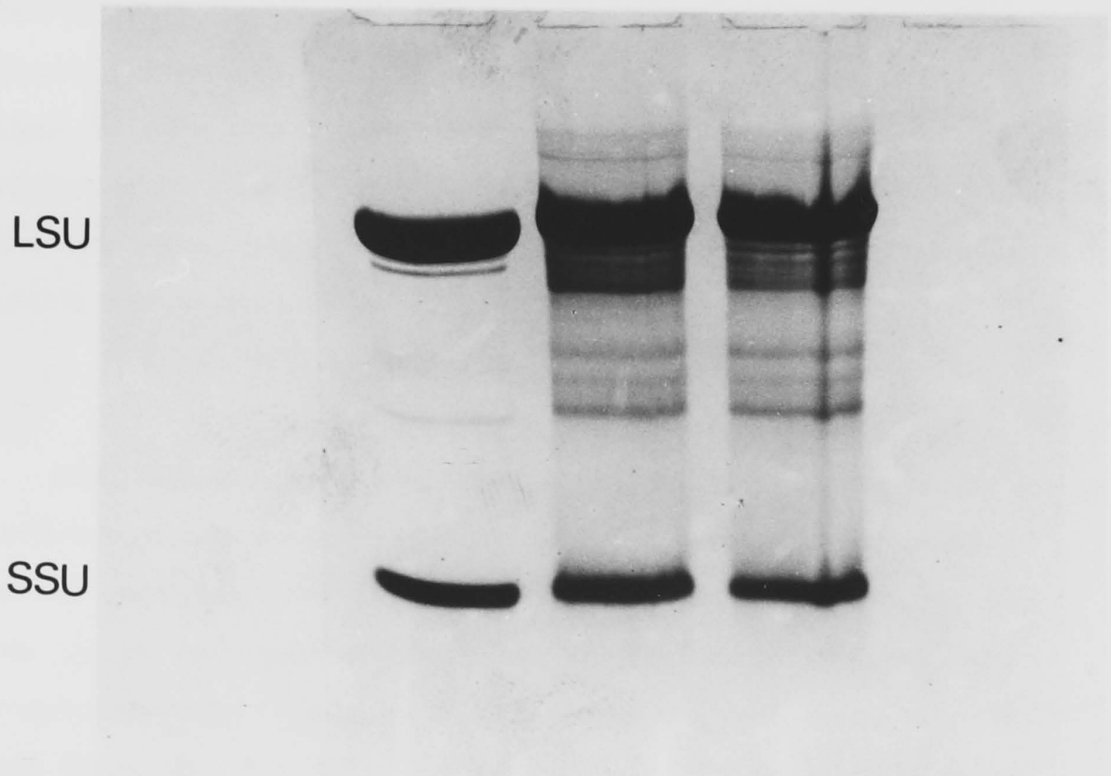
Fig. 6.3. SDS gel electrophoretograms of RuBPC-ase of HR, HP, and MF genotypes.

Isolation of RuBPC-ase of HR was carried out by Sepharose 6B column chromatography (Chapter 2.4.2). Separation of total leaf protein of HP and MF genotypes was by G25 Sephadex chromatography (Chapter 2.4.1). The peak fractions of RuBPC-ase and 'cytoplasmic' proteins were collected. 50-100 µg of protein was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, then loaded on a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA, and 0.1% SDS, pH 8.5. Electrophoresis was for 2h at 30 mA and approx. 120 V at room temperature. The gels were stained in Coomassie Blue solution for 4 h (Chapter 2.8.7). Destaining was done with a 6:1:13 mixture of ethanol, glacial acetic acid and water.

Lane 1: RuBPC-ase of the HR genotype.

Lane 2: Total leaf protein of the HP genotype.

Lane 3: Total leaf protein of the MF genotype.


LSU
SSU
1
2
3

6.2.2. (ii) Variation of the Ratio of LSU to SSU for HP, MF, Rambler and Du Puits Genotypes

The ratio of LSU to SSU was further investigated (Fig. 6.4) in two additional cultivars. One of these was Rambler (lane 2), a hybrid of M. sativa x M. falcata. The other was a high yielding French cultivar, Du Puits (M. sativa) (lane 4). HP (lane 1) and MF (lane 3) were also included for comparison. 100 µg of total leaf protein of the four genotypes was applied to a 13% SDS Davis gel for electrophoresis. No significant difference was found in the ratios of LSU to SSU for the four genotypes.

6.2.2. (iii) Variation in the Ratio of LSU to SSU for 'High' and 'Low' Protein Genotypes

High selection pressure had produced four genotypes with different protein contents (Chapter 5.2.1.). The total leaf proteins of the genotypes were first isolated by G25 column chromatography. 100 µg of each protein sample was supplemented with SDS and 2-mercaptoethanol, incubated at 50°C for 10 min, and applied to a 13% SDS Davis gel. Fig. 6.5 shows the results of SDS gel electrophoresis of the four proteins. Two 'high' protein genotypes (6.2, lane 2; 3.17, lane 4) and two 'low' protein genotypes (14.9, lane 1, 14, 29, lane 3) had a similar ratio of LSU to SSU. This indicated that high selection pressure did not change the ratio of LSU to SSU in selected genotypes.

Results described in this sub-chapter (6.2.2) showed that the LSU to SSU ratio was constant in both cultivars and selected genotypes when plants were grown under normal temperature conditions.

Fig. 6.4. SDS gel electrophoretograms of RuBPC-ase of HP, Rambler, MF, and Du Puits genotypes.

Total leaf proteins of the four genotypes were previously isolated by G25 column chromatography (Chapter 2.4.1). 100 µg aliquots of total leaf proteins from each of the four genotypes were supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), then heated at 50°C for 10 min before loading on a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA, and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx. 120 V at room temperature. The gels were stained in Coomassie Blue solution for 4 h (Chapter 2.8.7). Destaining was done with a 6:1:13 mixture of ethanol, glacial acetic acid and water.

Lane 1: Total leaf protein of HP genotype.

Lane 2: Total leaf protein of Rambler genotype.

Lane 3: Total leaf protein of MF genotype.

Lane 4: Total leaf protein of Du Puits genotype.

Fig. 6.5. SDS gel electrophoretograms of RuBPC-ase of 'high' and 'low' protein genotypes.

Total leaf protein of 'high' (6.2 genotype, 3.17 genotype) and 'low' (14.9 genotype, 14.29 genotype) selections were previously isolated by G25 column chromatography.

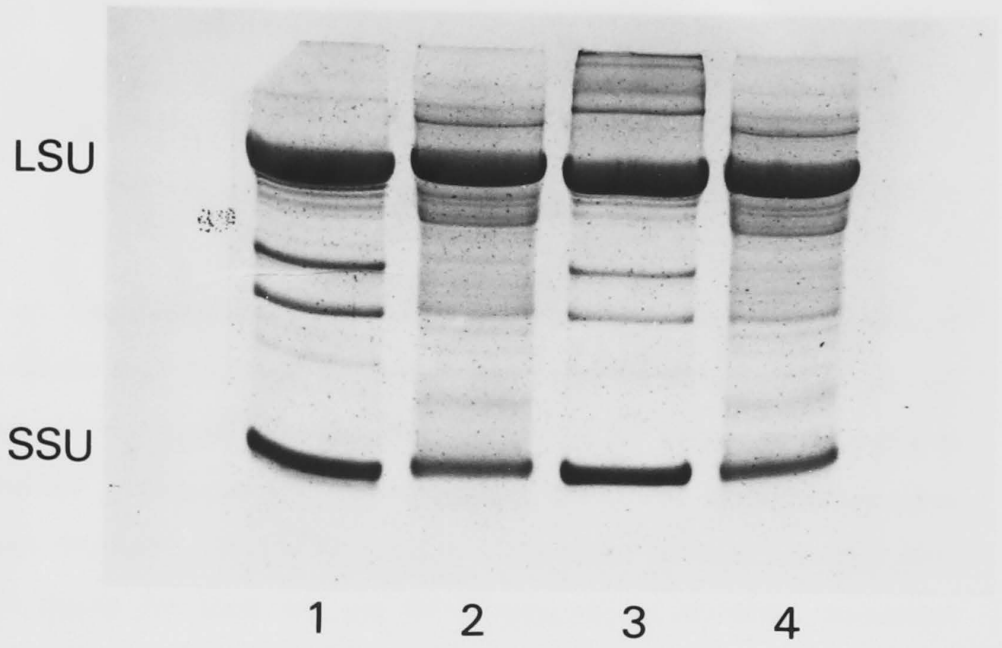
Samples were prepared for electrophoresis as described for Fig. 6.4.

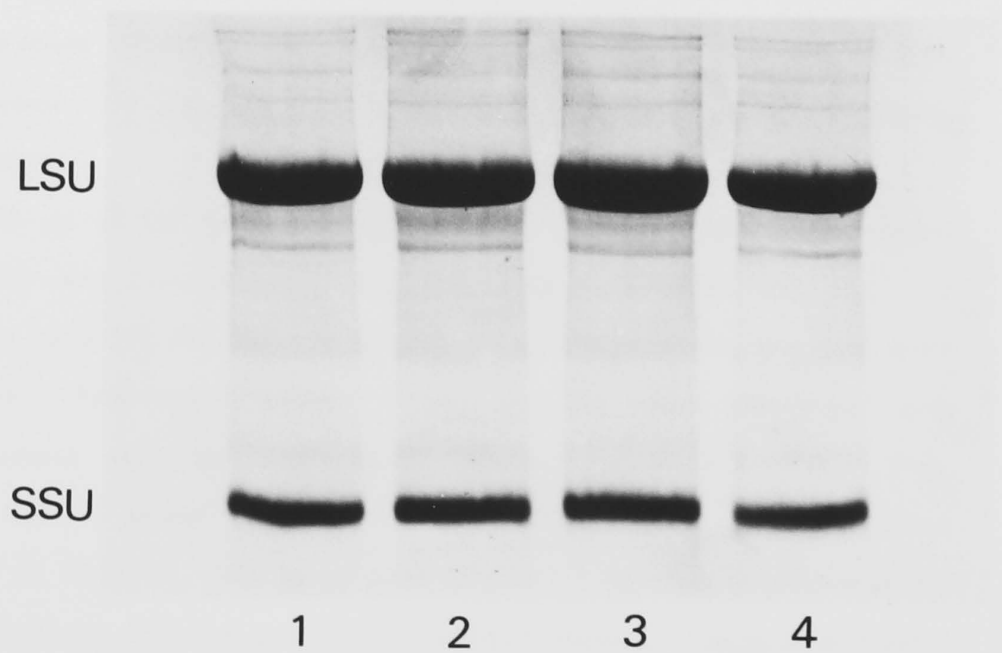
Lane 1: Total leaf proteins of (14) 9 genotype.

Lane 2: Total leaf proteins of (6) 2 genotype.

Lane 3: Total leaf proteins of (14) 29 genotype.

Lane 4: Total leaf proteins of (3) 17 genotype.


LSU
SSU
1
2
3
4


LSU
SSU
1
2
3
4

6.2.3. Effect of Temperature on Synthesis of LSU and SSU Polypeptides

6.2.3. (i) Effect of Two Temperature Regimes on Synthesis of Total Leaf Protein, LSU, and SSU

Two genotypes were grown at 25/19°C and 35/30°C day/night temperature and the total extracted leaf proteins were isolated by G25 column chromatography. The elution profiles of total leaf proteins of HP and MF genotypes are shown in Fig. 6.6. The proteins appeared between fractions 18 to 30, and the maximum protein concentration was 50 mg/ml for both HP and MF genotypes, at 25/19°C. Reduction of the protein concentration was apparent at 35/30°C in both HP (32 mg/ml) and MF (36 mg/ml) genotypes. The total protein yield of HP and MF was similar at 25/19°C (Table 6.1), and plant growth at high temperature (35/30°C) reduced the yield by 15% in the case of both genotypes. It was apparent that high temperature affected protein synthesis.

100 µg of the peak fraction for each genotype was supplemented by SDS and 2-mercaptoethanol, incubated at 50°C for 10 min, and applied to a 13% SDS gel. After 2 h of electrophoresis, the gels were stained. The track slices of one gel for each genotype (one replication) were scanned and the protein profile is shown in Fig. 6.7. Scan 1 shows the total protein profile for HP genotype plants grown at 25/19°C. The large peak is LSU (2 cm from origin) and the small peak is SSU polypeptide (6 cm from origin). There is a drastic reduction of the height of both polypeptide peaks, particularly the LSU, at 35/30°C (scan 2). The temperature had an opposite effect, as far as the MF genotype is concerned, on the synthesis of LSU and

Fig. 6.6. Chromatography elution profiles of the total leaf proteins of HP and MF genotypes.

Plants were grown in the phytotron at either 25/19°C or 35/30°C. The total leaf proteins were isolated by G25 column chromatography (Chapter 2.4.1), at a flow rate of 0.1 ml/min and eluted fraction volume of 1 ml.

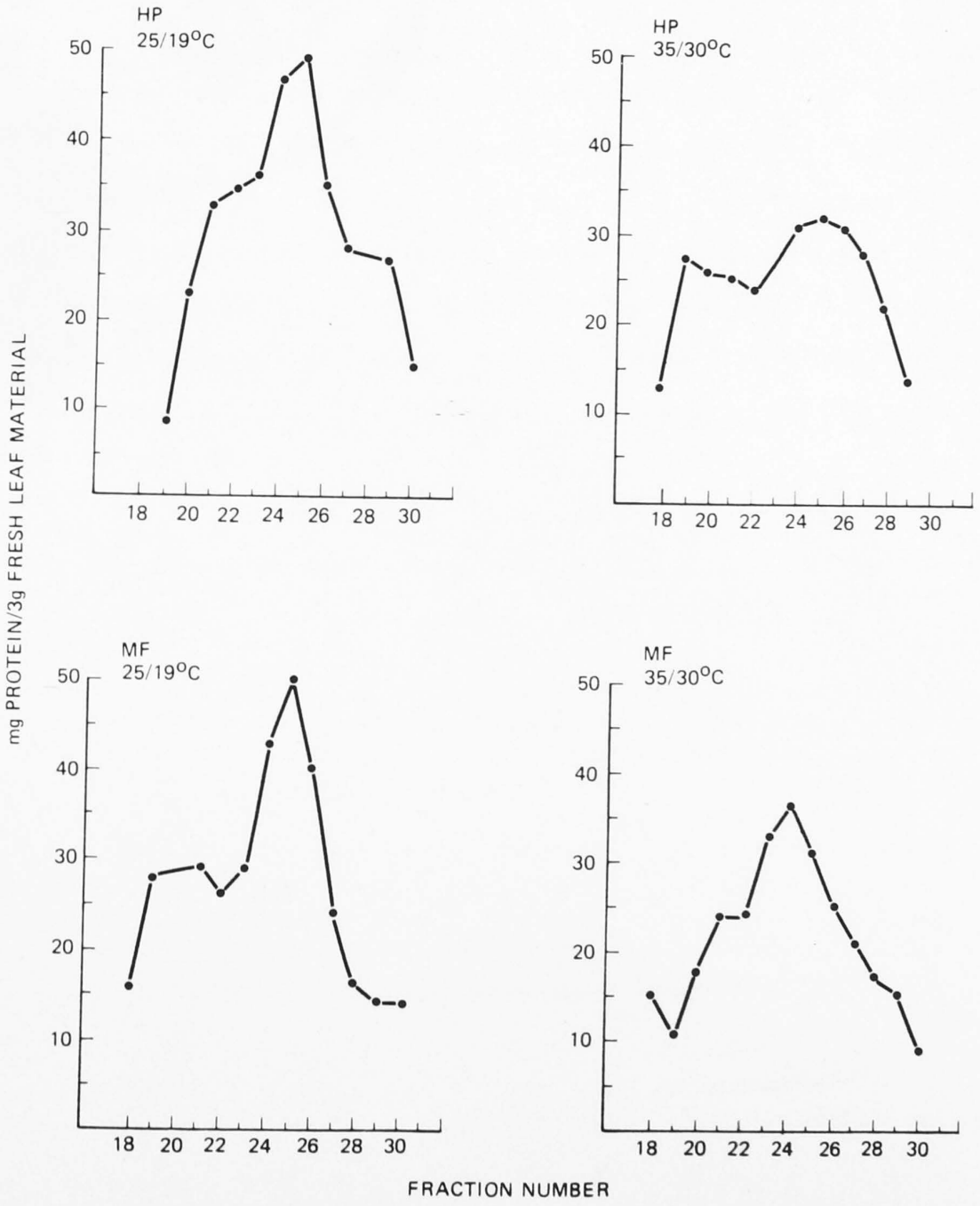

HP
25/19°C
HP
35/30°C
MF
25/19°C
MF
35/30°C
50
40
30
20
10
18 20 22 24 26 28 30
mg PROTEIN/3g FRESH LEAF MATERIAL
FRACTION NUMBER

Table 6.1. Effect of plant growth temperature on the total extracted leaf protein yields (mg/3 gm fresh leaves) of HP and MF genotypes.

Plants were grown in the phytotron at either 25/19°C or 35/30°C. The total leaf proteins were isolated by G25 column chromatography (Chapter 2.4.1.). Samples were dialysed against distilled water overnight and freeze dried.

Table 6.1. Effect of plant growth temperature on the total leaf protein yields (mg/3 gm fresh leaves) of HP and MF genotypes.

	Protein (mg)	
Temperature	HP	MF
25/19°C	335	329
35/30°C	283	281
Difference between protein yields	52	48

Fig. 6.7. Densitometric scan profile of total leaf proteins of HP and MF genotypes.

Plants were grown at two temperature regimes (25/19 and 35/30°C) and total leaf proteins were isolated by G25 column chromatography (Chapter 2.4.1). 100 µg of total leaf protein was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, then loaded on a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA, and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx. 120 V at room temperature. The gels were stained in Coomassie Blue solution for 4 h (Chapter 2.8.7). Destaining was done with a 6:1:13 mixture of ethanol, glacial acetic acid and water.

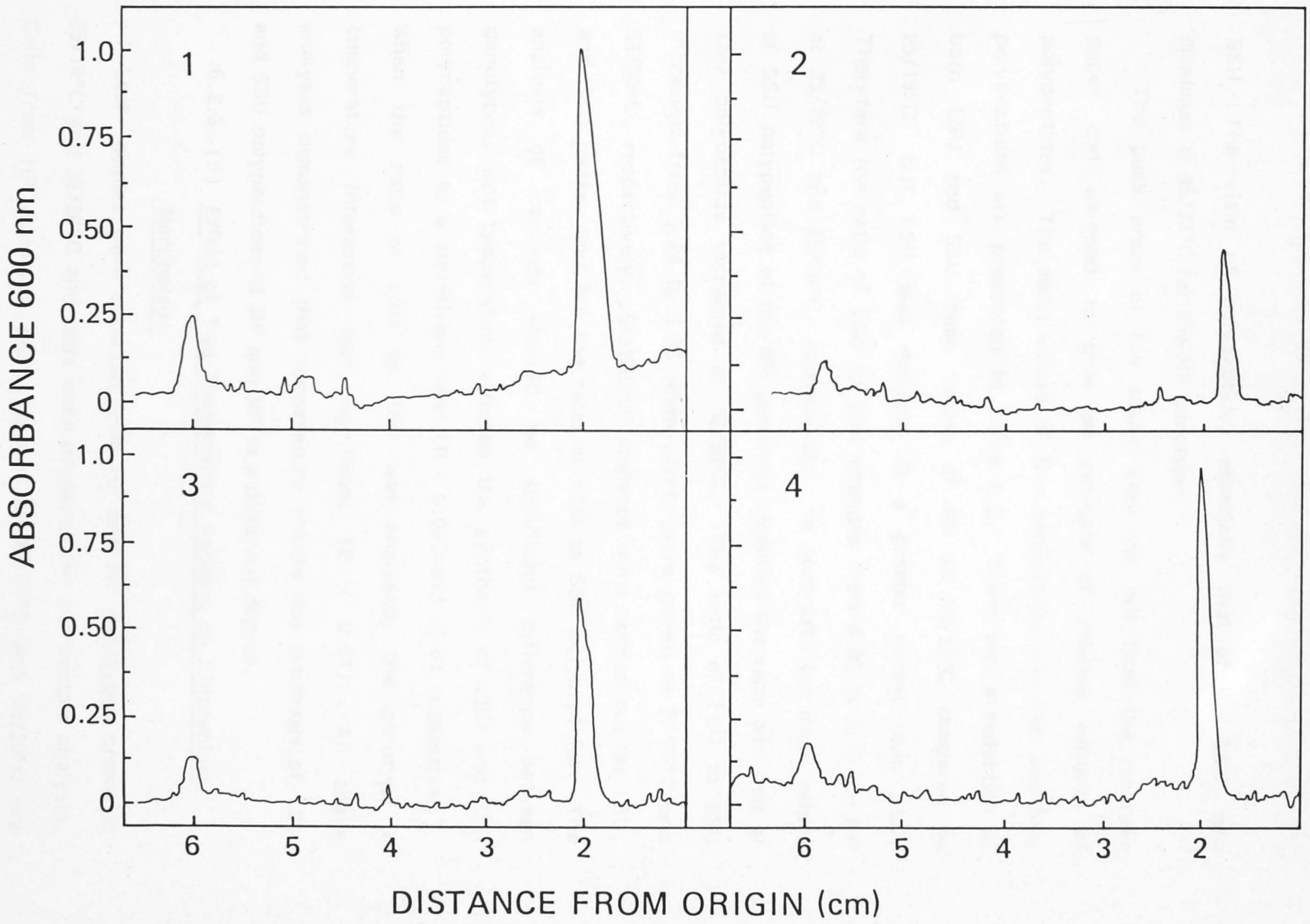
The gels were cut into slices 1 cm wide along the vertical direction of the gel and scanned with a Gilford spectrophotometer-240. The wave length was 600 nm and the absorbance control was 1500.

Scan 1: HP genotype at 25/19°C.

Scan 2: HP genotype at 35/30°C.

Scan 3: MF genotype at 25/19°C.

Scan 4: MF genotype at 35/30°C.


1
2
3
4
1.0
0.75
0.50
0.25
0
ABSORBANCE 600 nm
6
5
4
3
2
DISTANCE FROM ORIGIN (cm)

SSU. The yield of polypeptide, especially that of the LSU, was maximum at 35/30°C for the MF genotype.

The peak areas of the scans were cut out from the recorder paper and weighed to give an estimate of relative amounts of polypeptides. The mean values of five replications for LSU and SSU polypeptides are presented in Table 6.2. There was a reduction of both LSU and SSU mean values of HP at 35/30°C compared to 25/19°C, but LSU was reduced to a greater extent than SSU. Therefore the ratio of LSU to SSU changes from 4.80 to 3.33 for HP at 25/19°C and 35/30°C, respectively. In contrast, the mean values of SSU polypeptide of the MF genotype remained the same and that of LSU polypeptide increased at 35/30°C. The ratio of LSU to SSU increased from 3.24 to 5.35 when plants were grown at 25/19°C and 35/30°C, respectively. Statistical analyses were carried out for LSU, and SSU peaks, and for the ratio of LSU to SSU polypeptides. The analysis of variance showed no significant difference between genotypes, but temperature affected the synthesis of LSU and SSU polypeptides to a significant level ($P < 0.001$ and <0.01 respectively). When the ratio of LSU to SSU was analysed, the genotype x temperature interaction was significant ($P < 0.01$). All above analyses demonstrated that temperature affects the synthesis of LSU and SSU polypeptides of HP and MF to a different degree.

6.2.3. (ii) Effect of Two Temperature Regimes on Chloroplast Morphology

Leaf samples were collected from HP and MF genotypes grown at 25/19°C and 35/30°C and cells were prepared for microscopic analysis. Cells from HP genotype plants grown at 25/19°C and 35/30°C are shown in Fig. 6.8 (photo 1 and 3, respectively). The plastid areas

Table 6.2. Mean weight (mg) of recorder paper representing the area under LSU and SSU peaks of RuBPC-ase from HP and MF genotype plants, grown under different day/night temperature conditions.

LSU and SSU peaks of the densitometric profiles of 5x replicated experiments were cut out and the mean weights of the paper were used to calculate the LSU/SSU ratios.

Table 6.2. Mean weights (mg) of recorder paper representing the area under LSU and SSU peaks of RuBPC-ase from HP and MF genotype plants grown under different day/night temperature conditions.

(Means of five experiments)

Genotype	Temperature	LSU	SSU	Ratio
HP	25/19°C	17.6	3.9	4.80
	35/30°C	6.6	2.0	3.33
MF	25/19°C	8.6	2.8	3.24
	35/30°C	12.6	2.8	5.35

Fig. 6.8. Photomicrographs of cells of HP and MF genotype plants grown under two temperature regimes.

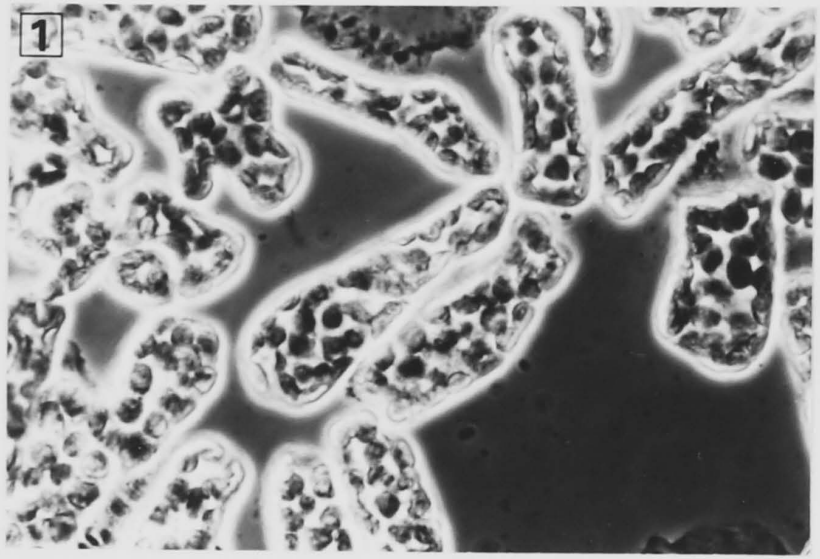
Plants of the two genotypes were grown at 25/19°C and 35/30°C. The leaf samples were collected and prepared for microscopic analysis as described in Chapter 2.10.

Photo 1: HP genotype at 25/19°C.

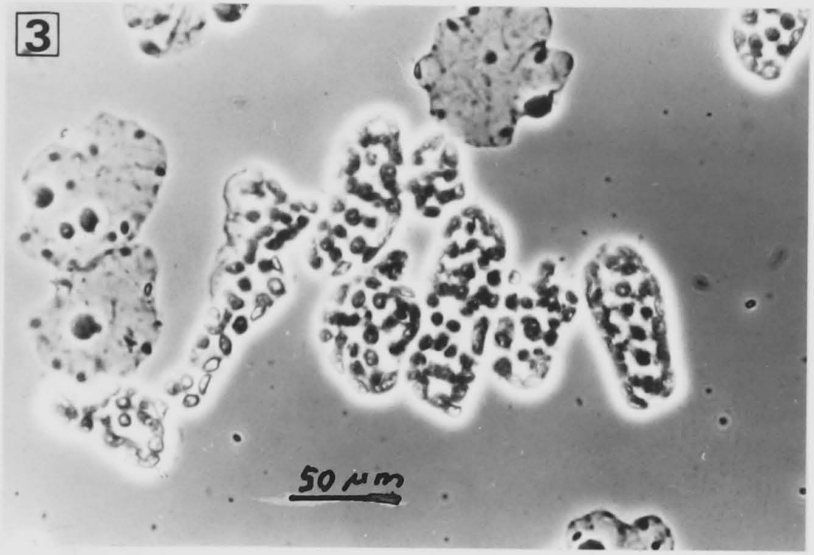
Photo 2: MF genotype at 25/19°C.

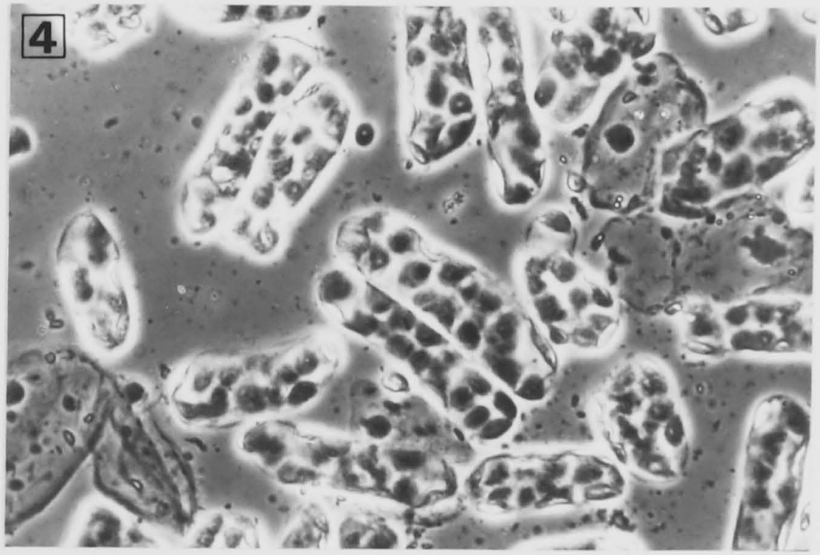
Photo 3: HP genotype at 35/30°C.

Photo 4: HP genotype at 35/30°C.


1


2


3
50 µm


4

at 25/19°C were "normal". In contrast, there was a marked reduction in plastid area when HP plants were grown at 35/30°C (photo 3) and gaps between the plastids increased. There was no apparent difference in plastid area of MF genotype cells from plants grown at the two temperatures.

Mean values of plastid number and plastid areas for HP and MF genotype plants grown at the two temperature regimes are presented in Table 6.3. The main finding is that the mean plastid area for cells of the HP genotype was greatly reduced.

Chloroplast ultrastructure, although not perfectly preserved in the electron micrographs of Fig. 6.9, can be seen to have been little affected by growth under the high temperature regime, 35/30°C. The HP genotype appears consistently to possess more starch than the MF at either temperature, but both genotypes produced healthy chloroplasts at either temperature.

6.2.4. Effect of Light Intensity on Synthesis of LSU and SSU Polypeptides

6.2.4. (i) Effect of Four Different Periods of Reduced Light Intensity on Total Leaf Protein

Plants of HP and MF genotypes were grown under reduced light intensity (12 μE $m^{-2}sec^{-1}$) for a period of one to four days. Total leaf proteins were extracted from the leaves and isolated by G25 column chromatography. The elution profile for the total protein solution of HP genotype plants is seen in Fig. 6.10. The protein eluted between fractions 19 to 26. The maximum concentration of total leaf proteins was 24 mg/ml after the first day, and 22 mg/ml after four days of growth. The total protein was 114 and 106 mg after one and four days, respectively. It is apparent that protein

Table 6.3. Mean plastid number and plastid area of HP and MF genotype plants grown under two temperature regimes.

Plants were grown at 25/19°C and 35/30°C temperatures. The leaf samples were collected and prepared for microscopic analysis (Chapter 2.10).

Table 6.3. Mean plastid number and plastid area of HP and MF genotype plants grown under two temperature regimes.

Genotype	Temperature	Plastids	
		No. per cell	Area (μm^2)
HP	25/19°C	20.8	13.14
HP	35/31°C	24.8	5.07
MF	25/19°C	29.3	16.91
MF	35/31°C	29.3	12.68

Fig. 6.9. Electron micrograph of mesophyll chloroplasts of HP and MF genotypes of Medicago grown under two different temperature regimes, 25/19°C and 35/30°C.

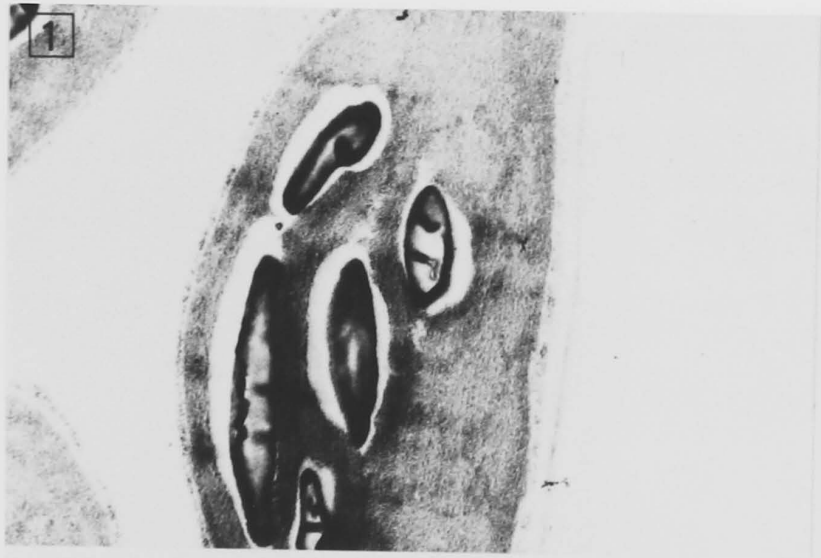
In all cases, leaf tissues were fixed in 2.5% glutaraldehyde in 35 mM Na PIPES buffer, pH 8, 5 mM $MgCl_2$ and left overnight at 4°C. Fixed tissues were washed in PIPES buffer (50 mM, pH 6.8), post-fixed in $0s0_4$ (1:1 mixture of 5% aq. $0s0_4$ solution and 50 mM Na PIPES buffer, pH 6.5), washed in dist. H_20, and dehydrated using an ethanol concentration series. Infiltration and embedding in Spurr's Resin A (film) (Spurr 1969) were done using propylene oxide. Sections were cut on a Reichert 0MU3 ultramicrotome, grid-stained using 2% barium permanganate (Pyliotis 1974), and examined in a JEOL 100C electron microscope. Magnification = 8500 x.

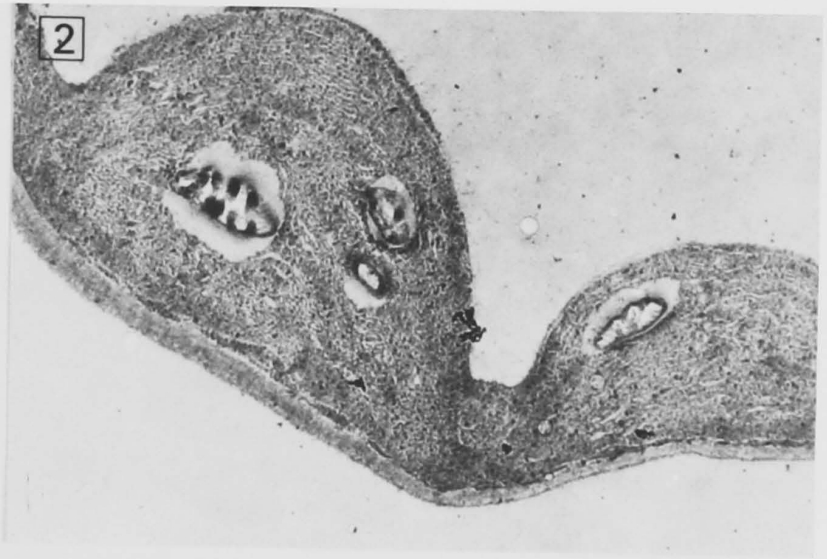
Photo 1 HP at 35/30°C

Photo 2 MF at 35/30°C

Photo 3 HP at 25/19°C

Photo 4 MF at 25/19°C


1

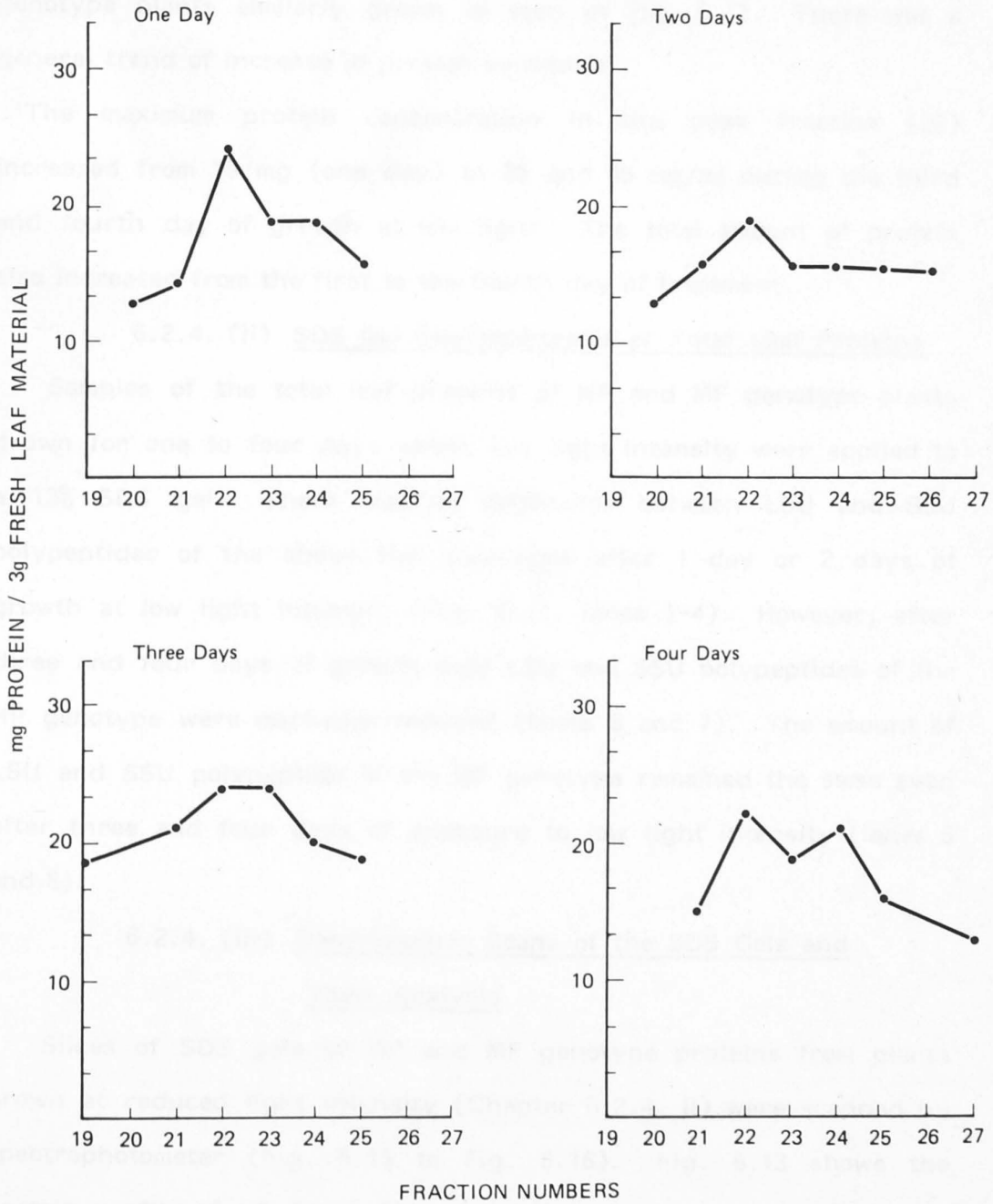

2


3


4

Fig. 6.10. Chromatography elution profiles for total leaf proteins of HP genotype plants grown under reduced light intensity.

Plants were grown for four different time periods at reduced light intensity (12 $\mu E\ m^{-2}\ sec^{-1}$). Leaf proteins were extracted from plants and further purified by G25 column chromatography (Chapter 2.4.1). The elution rate was 0.1 ml/min and the protein content of each sample was determined.


One Day
Two Days
Three Days
Four Days
30
20
10
19 20 21 22 23 24 25 26 27
mg PROTEIN / 3g FRESH LEAF MATERIAL
FRACTION NUMBERS

synthesis in HP genotype plants decreased only slgithly during the four-day period. The elution profile for total proteins of MF genotype plants similarly grown is seen in Fig. 6.11. There was a general trend of increase in protein synthesis.

The maximum protein concentration in the peak fraction (22) increased from 19 mg (one day) to 35 and 45 mg/ml during the third and fourth day of growth at low light. The total amount of protein also increased from the first to the fourth day of treatment.

6.2.4. (ii) SDS Gel Electrophoresis of Total Leaf Proteins

Samples of the total leaf proteins of HP and MF genotype plants grown for one to four days under low light intensity were applied to a 13% SDS gel. There was no difference between LSU and SSU polypeptides of the above two genotypes after 1 day or 2 days of growth at low light intensity (Fig. 6.12, lanes 1-4). However, after three and four days of growth both LSU and SSU polypeptides of the HP genotype were markedly reduced (lanes 5 and 7). The amount of LSU and SSU polypeptide of the MF genotype remained the same even after three and four days of exposure to low light intensity (lanes 6 and 8).

6.2.4. (iii) Densitometric Scans of the SDS Gels and Their Analysis

Slices of SDS gels of HP and MF genotype proteins from plants grown at reduced light intensity (Chapter 6.2.4. ii) were scanned by spectrophotometer (Fig. 6.13 to Fig. 6.16). Fig. 6.13 shows the protein profile of HP (scan 1) and MF (scan 2) genotypes. The LSU polypeptide is located at 2 cm and the SSU polypeptide is at 6 cm from the origin. The LSU and SSU of the HP genotype (scan 1) are present in smaller amount but at the "normal" ratio. The polypeptides of the MF genotype (scan 2) are synthetized at a normal

Fig. 6.11. Chromatography elution profiles for total leaf proteins of MF genotype plants grown under reduced light intensity.

Plants were grown for four different time periods at reduced light intensity. Leaf proteins were extracted from plants and further purified by G25 column chromatography (Chapter 2.4.1). The elution rate was 0.1 ml/min and the protein content of each sample was determined.

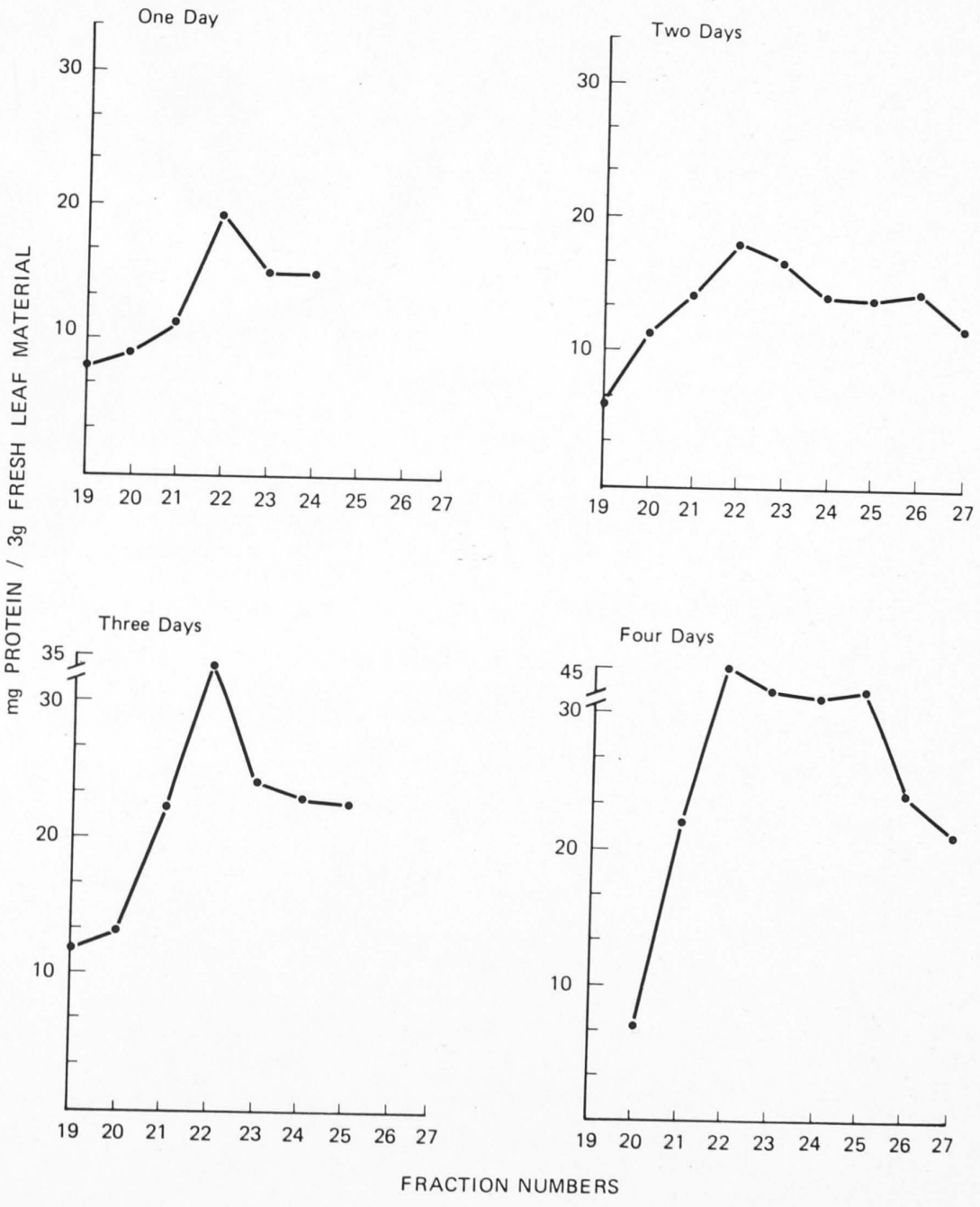
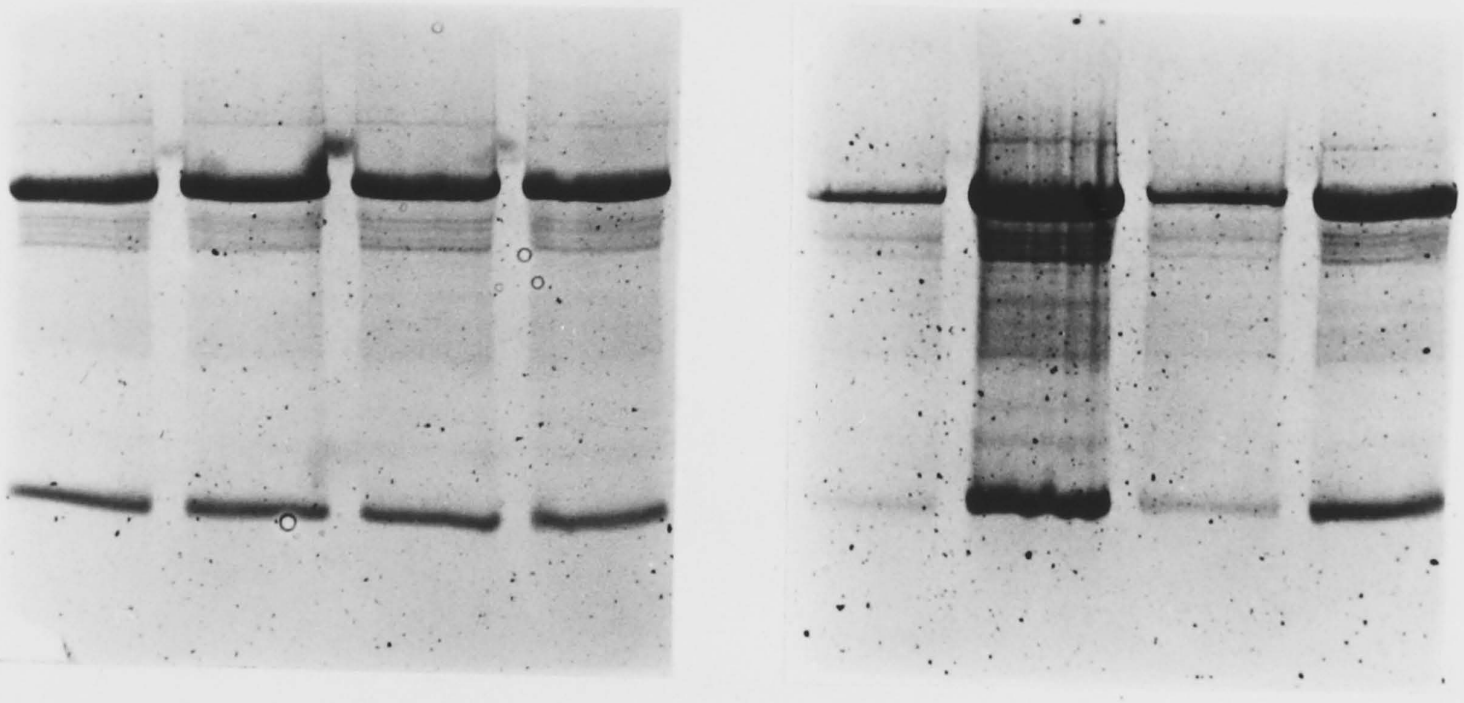

One Day
Two Days
Three Days
Four Days
mg PROTEIN / 3g FRESH LEAF MATERIAL
FRACTION NUMBERS
10
20
30
35
45
19 20 21 22 23 24 25 26 27

Fig. 6.12. SDS gel electrophoretograms of total leaf proteins from plants of two different genotypes, grown under low light intensity for one to four days.

The light intensity was 12 mE m^{-2} sec^{-1}. 100 µg samples of proteins were supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50°C for 10 min, then loaded on a 13% SDS Davis gel (Chapter 2.5.3). The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA and 0.1% SDS, pH 8.5. Electrophoresis was for 2 h at 30 mA and approx. 120 V at room temperature. The gels were stained in Coomassie Blue solution for 4 h (Chapter 2.8.7). Destaining was done with a 6:1:13 mixture of ethanol, glacial acetic acid and water.

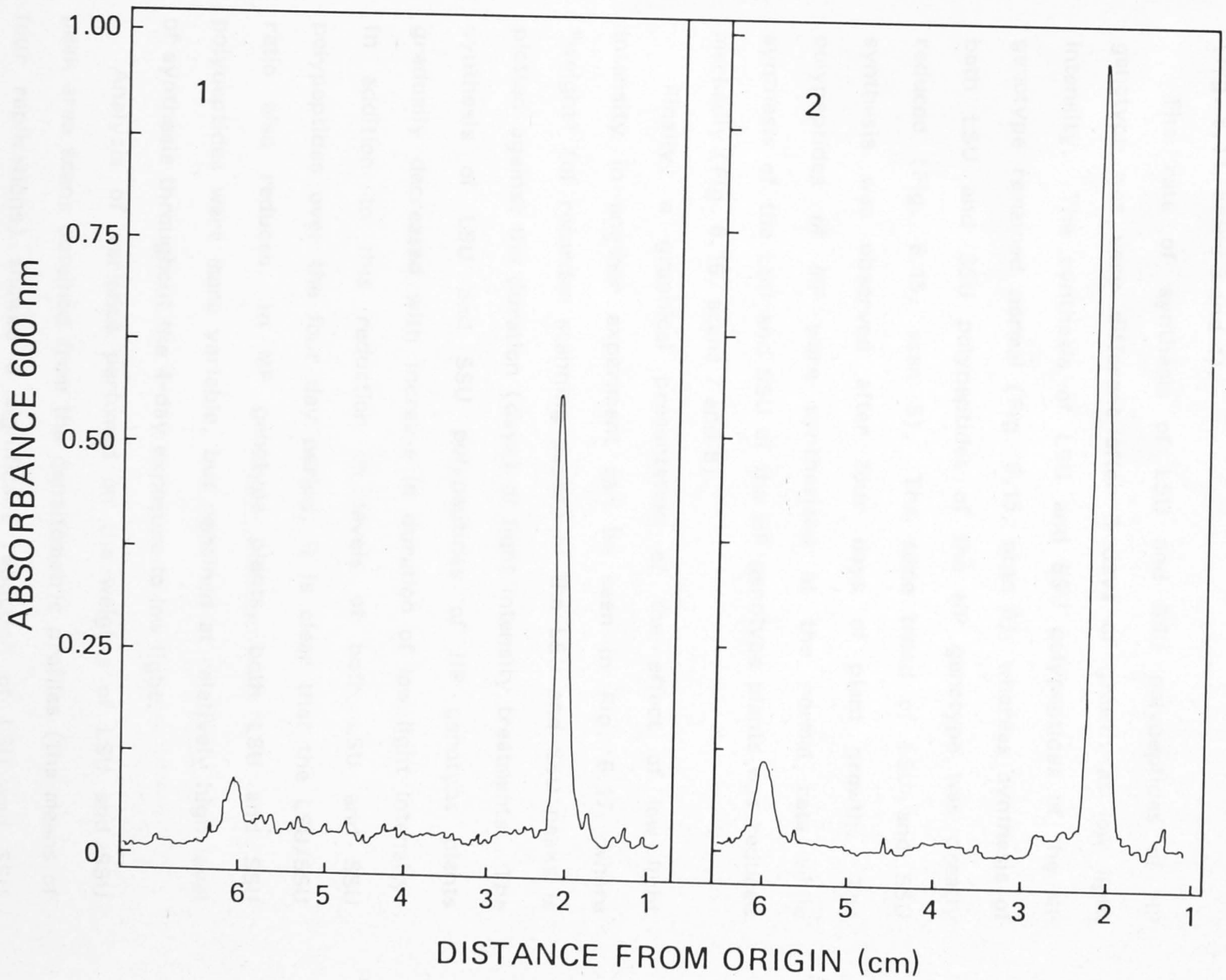
Lanes 1 and 2 of the gel represent total proteins of HP and MF genotype plants grown 1 day at low light intensity. Lanes 3 to 8 represent the same genotype-paired comparison for plants grown under low light for 2,3, and 4 days, respectively.



1 2 3 4 5 6 7 8

Fig. 6.13. Densitometric scan profile of total leaf proteins of HP (scan 1) and MF (scan 2) genotype plants grown at low light intensity for 1 day.

The gel was prepared as described in Fig. 6.12. The stained gel was cut into slices (1 cm wide) in the vertical direction of the gel and scanned with a Gilford spectrophotometer 240. The wave length was set at 600 nm and the absorbance control at 1500.


1.00
0.75
0.50
0.25
0
ABSORBANCE 600 nm
1
2
6
5
4
3
2
1
6
5
4
3
2
1
DISTANCE FROM ORIGIN (cm)

rate during plant growth for one day at low light intensity. The subunit polypeptides of both HP and MF genotypes are synthesized at the normal rate also during the second day under low light intensity (Fig. 6.14, scans 3 and 4).

The rate of synthesis of LSU and SSU polypeptides of HP genotype was very different after 3 days of growth at low light intensity. The synthesis of LSU and SSU polypeptides of the MF genotype remained normal (Fig. 6.15, scan 6), whereas synthesis of both LSU and SSU polypeptides of the HP genotype was greatly reduced (Fig. 6.15, scan 5). The same trend of LSU and SSU synthesis was observed after four days of plant growth. The polypeptides of MF were synthesized at the normal rate while synthesis of the LSU and SSU of the HP genotype plants was reduced markedly (Fig. 6.16, scans 7 and 8).

Finally, a graphical presentation of the effect of low light intensity in another experiment can be seen in Fig. 6.17, where "weight" (of recorder scanning paper) of the LSU and SSU peaks is plotted against the duration (days) of light intensity treatments. The synthesis of LSU and SSU polypeptides of HP genotype plants gradually decreased with increase in duration of low light intensity. In addition to this reduction in levels of both LSU and SSU polypeptides over the four day period, it is clear that the LSU/SSU ratio also reduces. In MF genotype plants, both LSU and SSU polypeptides were more variable, but remained at relatively high level of synthesis throughout the 4-day exposure to low light.

Analysis of variance perfomed on the weights of LSU and SSU peak area scans obtained from the densitometric profiles (the means of four replications) showed a significant reduction of LSU and SSU

Fig. 6.14. Densitometric scans of total leaf proteins of HP (scan 3) and MF (scan 4) genotype plants grown at low light intensity for two days.

The gel was prepared according to Fig. 6.12. The stained gel was cut into slices 1 cm wide in the vertical direction of the gel and scanned with a Gilford spectrophotometer 240. The wave length was set at 600 nm and the absorbance control at 1500.

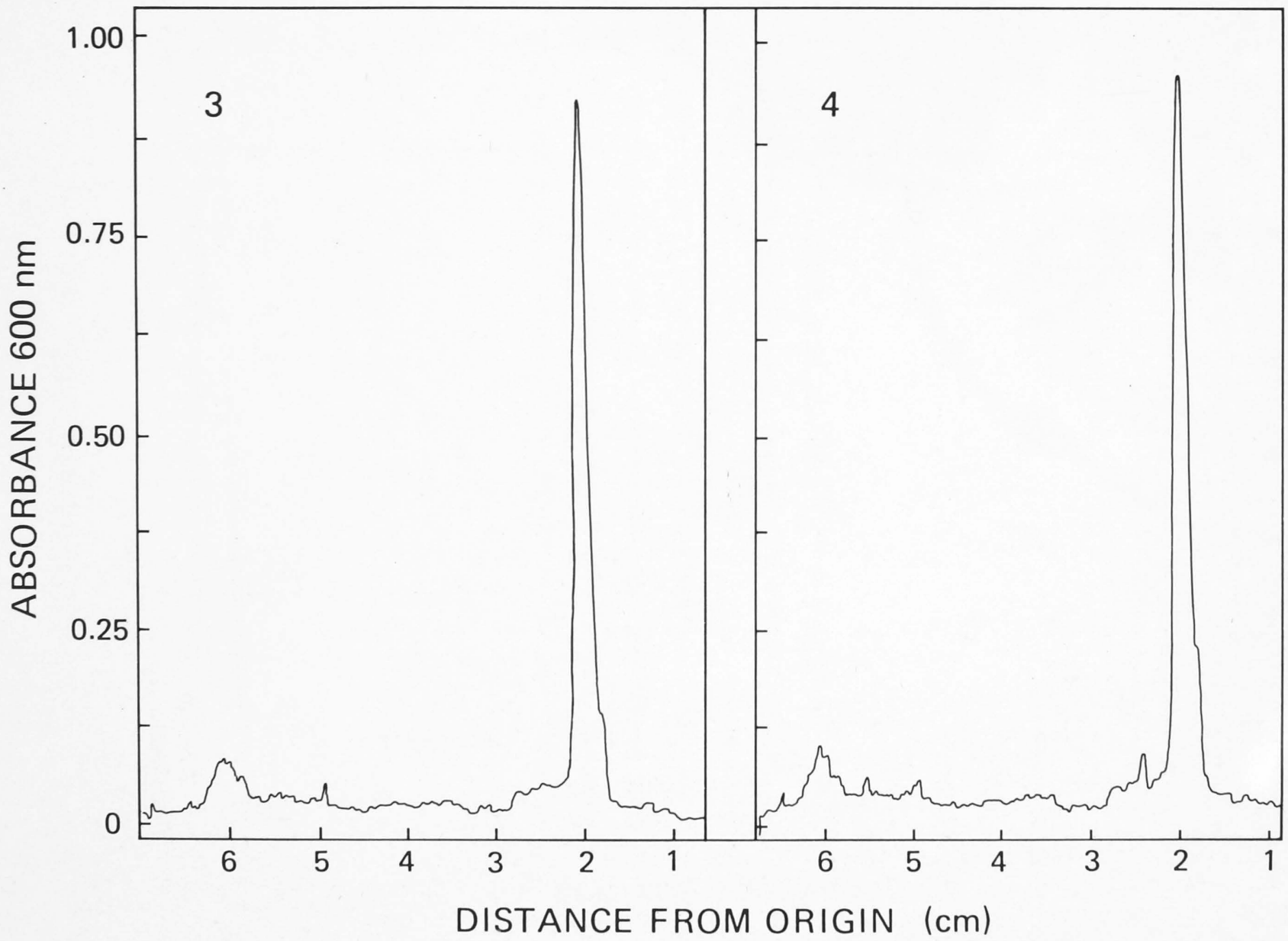

1.00
0.75
0.50
0.25
0
ABSORBANCE 600 nm
3
4
6
5
4
3
2
1
6
5
4
3
2
1
DISTANCE FROM ORIGIN (cm)

Fig. 6.15. Densitometric scan profile of total leaf proteins of HP (scan 5) and MF (scan 6) genotype plants grown at low light intensity for 3 days.

The gels were prepared as described in Fig. 6.12. The stained gel was cut (1cm wide slices) in the vertical direction of the gel and scanned with a Gilford spectrophotometer 240. The wave length was set at 600 nm and the absorbance control at 1500.

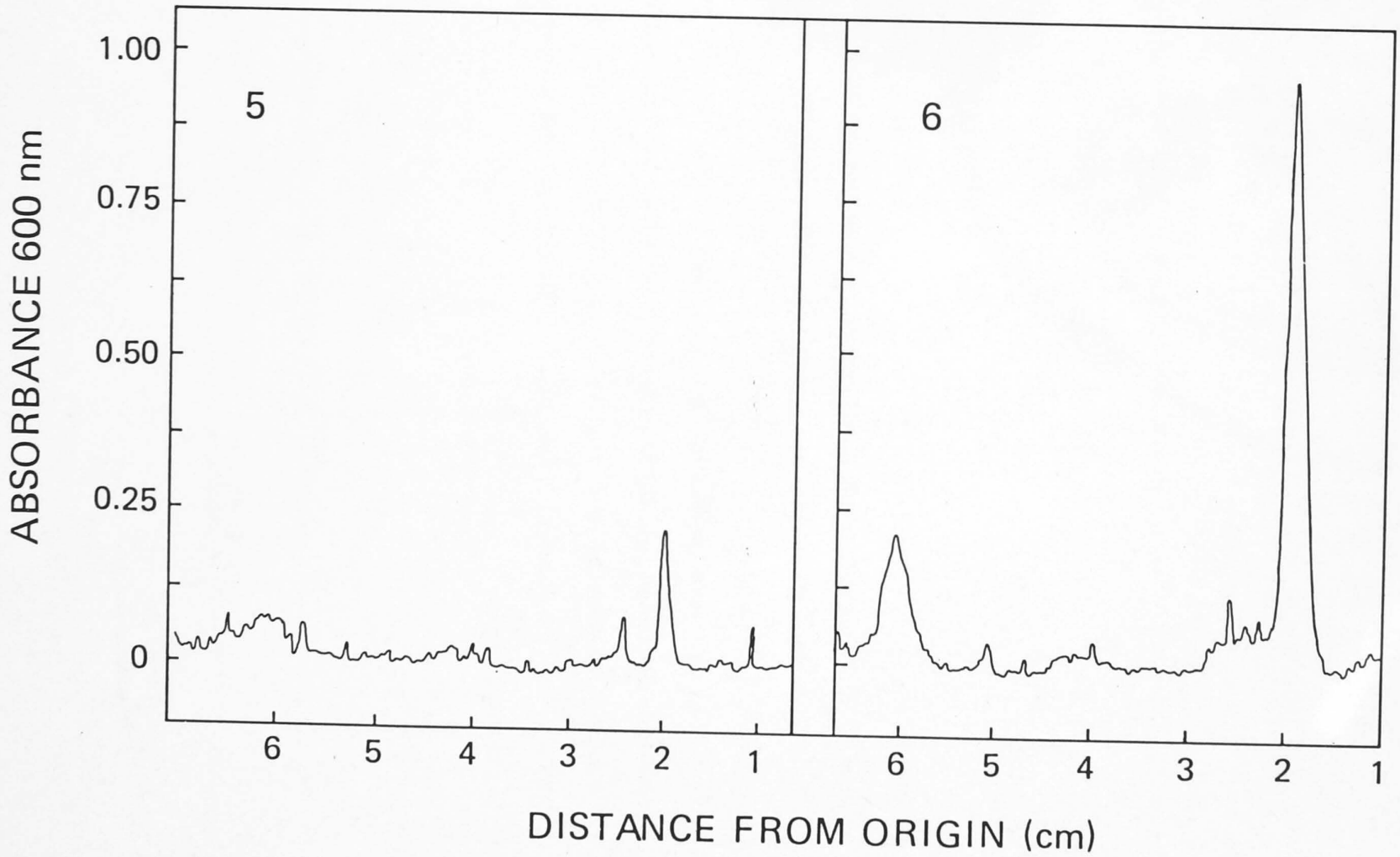

ABSORBANCE 600 nm
1.00
0.75
0.50
0.25
0
5
6
6 5 4 3 2 1
6 5 4 3 2 1
DISTANCE FROM ORIGIN (cm)

Fig. 6.16. Densitometric scan profile of total leaf proteins of HP (scan 7) and MF (scan 8) genotype plants grown at low light intensity for 4 days.

The gel was prepared as described in Fig. 6.12. The stained gel was cut into slices (1 cm wide) in the vertical direction of the gel and scanned by a Gilford spectrophotometer 240. The wave length was set at 600 nm and the absorbance control at 1500.

ABSORBANCE 600 nm

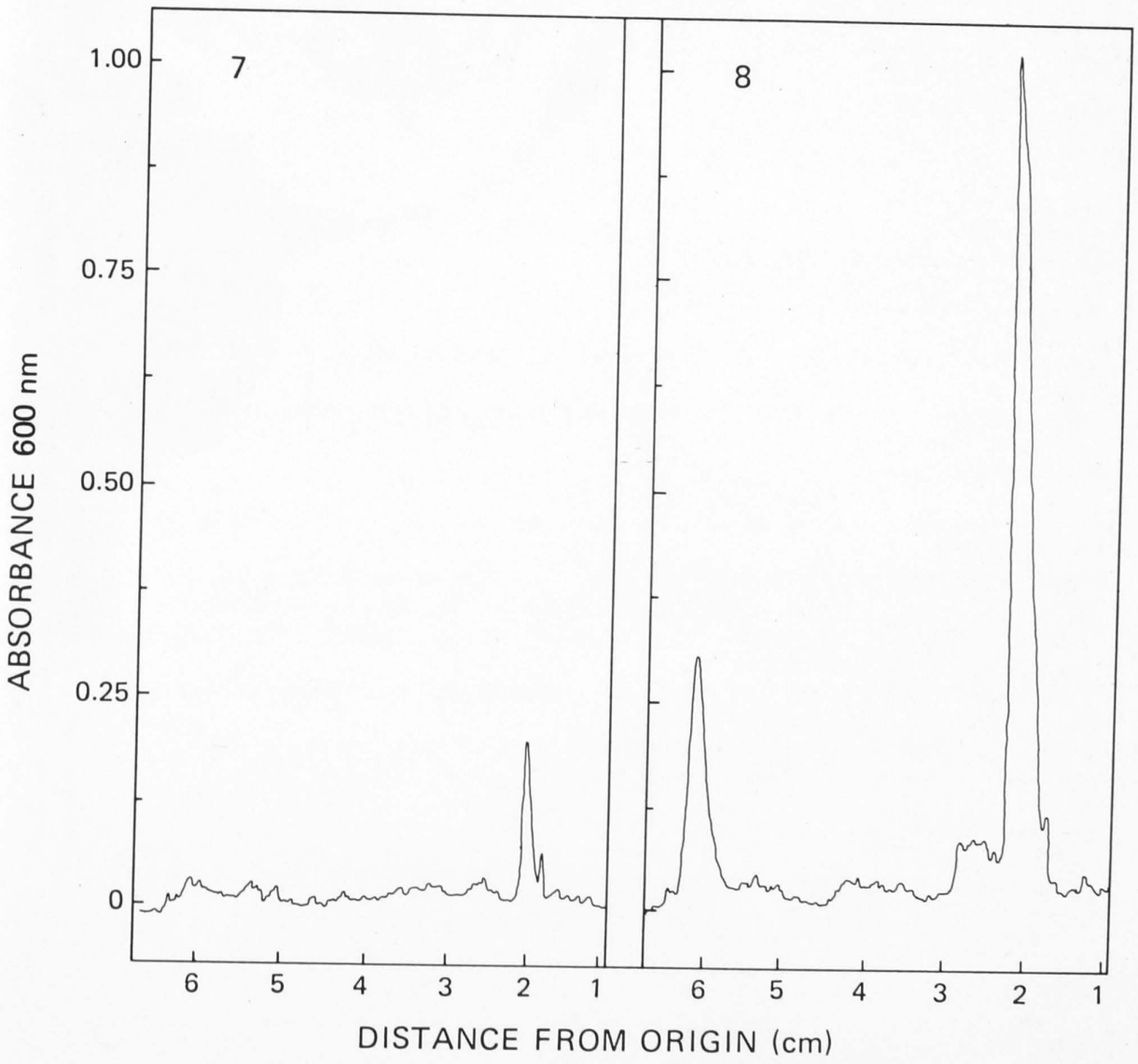
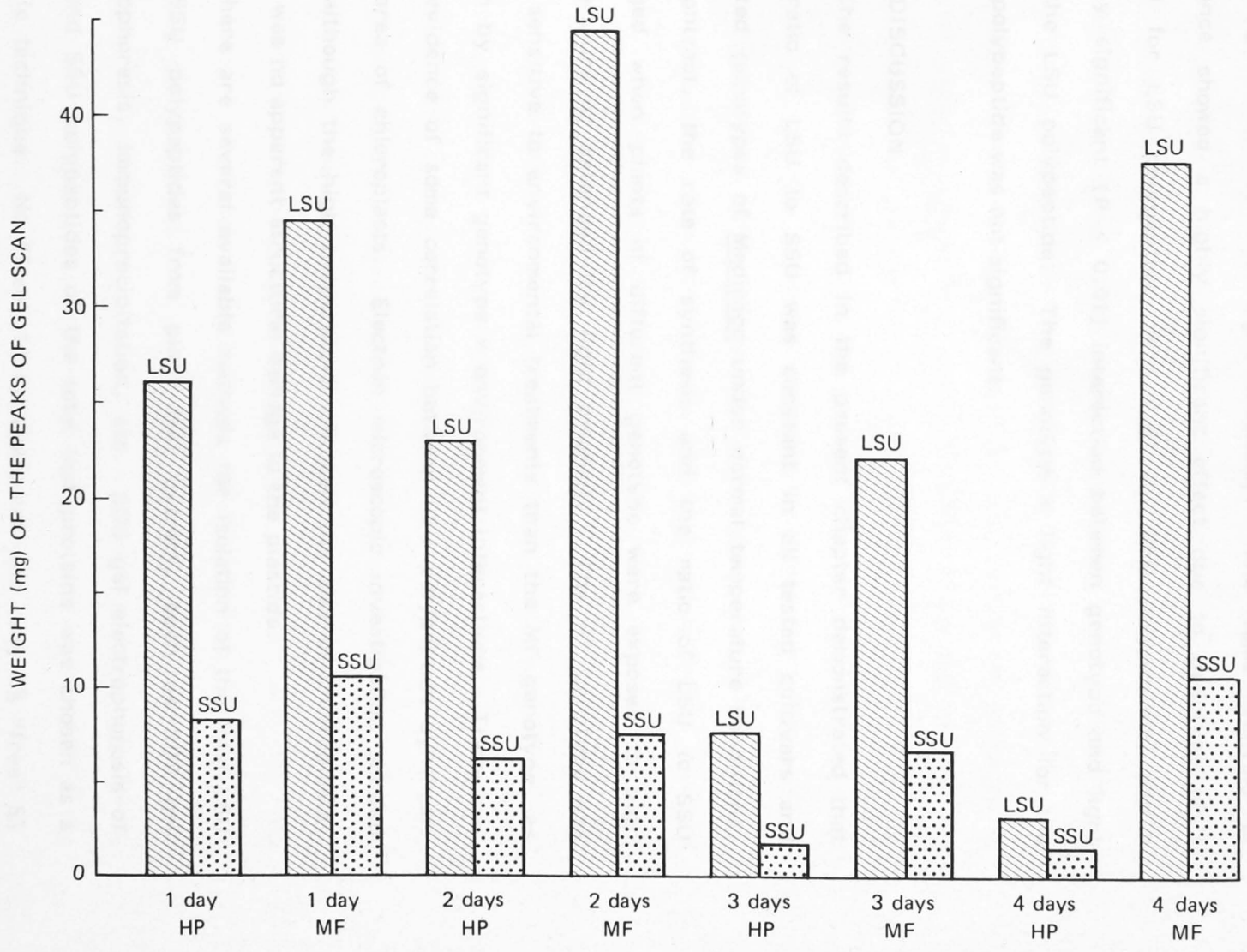

7
8
1.00
0.75
0.50
0.25
0
ABSORBANCE 600 nm
6
5
4
3
2
1
6
5
4
3
2
1
DISTANCE FROM ORIGIN (cm)

Fig. 6.17. Histogram of the relative amounts of LSU and SSU polypeptides of HP and MF genotype plants grown under reduced light intensity.

The plants were grown for periods of one to four days at low light intensity (12 μE m^{-2} sec^{-1}). The values represent the mean weights (weight of recorder scan paper for peak areas)obtained from analysis of densitometric scans of gels (Chapter 2.12).

Effect of four different durations of reduced light intensity on the synthesis of LSU and SSU of HP and MF genotypes



polypeptides of the HP genotype after the third and fourth day of plant growth under low light intensity. The same analysis of variance showed a highly significant effect due to genotype ($P < 0.001$ for LSU polypeptide, $P < 0.01$ for SSU polypeptide) and a highly significant ($P < 0.01$) interaction between genotype and light for the LSU polypeptide. The genotype x light interaction for the SSU polypeptide was not significant.

6.3 DISCUSSION

The results described in the present chapter demonstrated that the ratio of LSU to SSU was constant in all tested cultivars and selected genotypes of Medicago under normal temperature conditions. In contrast, the rate of synthesis and the ratio of LSU to SSU changed when plants of different genotype were exposed to high temperature or extended low light intensity. The HP genotype was more sensitive to environmental treatments than the MF genotype, as shown by significant genotype x environment interactions. There was also evidence of some correlation between LSU polypeptide synthesis and area of chloroplasts. Electron microscopic investigation showed that although the high temperature reduced the size of chloroplasts, there was no apparent structural damage to the plastids.

There are several available methods for isolation of the total LSU and SSU polypeptides from plant leaf extract, such as SDS gel electrophoresis, immunoprecipitation, etc. SDS gel electrophoresis of LSU and SSU polypeptides of the total leaf proteins was chosen as a suitable technique. No "free" 14 kd molecules and only 8% "free" 53 kd molecules (probably LSU polypeptides) were found in the 'cytoplasmic' proteins. The method of immunoprecipitation may have

some associated problems. Gray and Kekwick (1973, 1974) examined the polypeptide released from 80S cytoplasmic ribosomes of green leaves following incubation with radioactive amino acids and high speed supernatant enzymes of bean leaves and rat liver. Of the polypeptides released, 30% were precipitated by antiserum to RuBPC-ase SSU whereas only 6% were precipitated with LSU antiserum. The protein precipitated by SSU antiserum appeared to coincide with "standard" SSU on gel filtration, while that precipitated by LSU antiserum also contained about 50% SSU. However, when the polysomes themselves, with their nascent polypeptides attached, were reacted with the two antisera, the anti-LSU serum precipitated 14% of the polyribosomes, while antiserum to the SSU precipitated only 4%. They postulated a pool of free LSU molecules which became attached to the nascent SSU polypeptides before their release from the polyribosome. Alscher et al. (1976) carried out similar experiments on polysomes from greening barley leaves, using a 100,000 g supernatant from E. coli as a source of accessory components. They found that this system specifically translated via chloroplast polysomes and that immunoprecipitation and peptide mapping of the products indicated that the LSU of RuBPC-ase was a major product. They concluded that the mRNA for the LSU was associated with the 70S chloroplast ribosomes. Roy et al. (1976) studied the immunoprecipitation of the radioactive polypeptides released from greening wheat leaf polysomes in the presence of a 30,000 g supernatant fraction prepared from wheat germ. They found that the chloroplast polyribosomes were inactive in this system. Roy et al. also reported that this system was inhibited up to 80% by 10 µg/ml cycloheximide, whereas the system used by Gray and Kekwick was only sensitive to the extent of 18% at a cycloheximide concentration of 100 µg/ml. Precipitation of the product with antiserum to the SSU of

RuBPC-ase followed by SDS-acrylamide electrophoresis resulted in a peak of activity coincident with the SSU. They presented arguments based on the consideration of a number of parameters, including tryptic peptide mapping, to conclude that the SSU is synthesised on the 80S polyribosomes.

Translation of the poly-A containing fraction of RNA from Chlamydomonas in a wheat germ system, followed by immunoprecipitation with antibodies to the SSU of RuBPC-ase, resulted in the isolation of a polypeptide which was about 3.5 kd longer than the SSU (Dobberstein et al. 1977). This precursor was reduced to the correct size of the SSU by a polysomal endoprotease from Chlaymdomonas. They postulate that the extra length of polypeptide may cause the translating polyribosome complex to attach to the outer chloroplast membrane where it would be cut down to size before becoming associated with the large subunits being synthesised within the chloroplast. It may be significant that Roy et al. (1976) also found a larger polypeptide from the cytoplasmic polysomes of wheat which was precipitated by the antiserum to the SSU.

Although the ratio of LSU to SSU in my experiments with Medicago was similar in standard cultivars and also in 'high' and 'low' leaf protein genotypes under normal environmental conditions, the extreme conditions (high temperature, low light intensity) were able to change the co-ordinated synthesis of LSU and SSU. The high temperature reduced the total leaf proteins of the HP and the MF genotypes. The amount of both LSU and SSU polypeptides of the HP genotype was reduced at 35/30°C, but the reduction of LSU polypeptide was greater. The effect of high temperature (32°C) was markedly different on the formation of SSU and LSU polypeptides of

young rye plants (Secale cereale L.) (Feierabend and Mikus, 1977). RuBPC-ase was not detected in extracts of 32°C-grown rye leaves that were unable to perform chloroplast protein synthesis because of a high-temperature-induced deficiency of 70S ribosomes. The LSU was absent and the SSU polypeptide was synthesized by cytoplasmic ribosomes in young rye leaves at 32°C (Feierabend and Wildner, 1978). These results clearly show that the translation of SSU of RuBPC-ase was not closely coupled by a co-ordinate control with that of LSU synthesis, and accumulation of the SSU occurred even in the complete absence of the LSU. The marked difference between the results of Feierabend and Mikus and the present investigation is that LSU synthesis was completely eliminated and SSU synthesis remained normal in leaves of rye at 32°C. In contrast, both LSU and SSU synthesis were reduced, although the reduction in LSU was greater than that in SSU of the HP genotype in my experiment. Both experiments were similar in the absence of co-ordinate control of LSU and SSU synthesis of RuBPC-ase at high temperature.

Genotype x temperature interaction for LSU and SSU synthesis was highly significant in Medicago. No other information has been reported concerning genotype x temperature interaction of LSU and SSU in other plant species.

LSU polypeptide synthesis was correlated with chloroplast area at different temperatures in Medicago. No similar experimental results appear to have been published.

Reduced light intensity resulted in a significant decrease of both subunits of RuBPC-ase of HP genotype but the reduction was markedly greater for LSU than for SSU synthesis. Similar reduction of the subunits of RuBPC-ase in darkness was reported for Lemna

gibba L. (Tobin and Suttie, 1980). The incorporation of ^{35}S methionine into protein was used to determine the relative decrease of subunit synthesis in this aquatic species. However, since the amino acid composition of the two subunits of RuBPC-ase from Lemna was not known, the ratio of methionine in the two subunits could not reliably be used to compare synthesis rates for each subunit. Therefore the ratio of LSU to SSU polypeptides in the present investigation should give a more accurate picture of changes in subunit ratios of genotypes in Medicago. No genotype x light intensity interaction has been reported previously.

An important consideration in photosynthesis is the mechanism of increase or decrease of subunit synthesis by light. Tobin (1978) reported that light and dark can affect the synthesis of RuBPC-ase and the mRNA for the SSU of RuBPC-ase in L. gibba. The synthesis of RuBPC-ase and the level of translatable mRNA for its SSU dropped to a very low level when green plants were transferred to the dark for 4 days. When these plants were returned to light for 18 h, the amount of translatable RNA for SSU of RuBPC-ase increased relative to other mRNA.

The effect of light on levels of mRNA coding for SSU of RuBPC-ase in pea seedlings was reported by Sasaki et al. 1981. The level of mRNA or RuBPC-ase and the rate of enzyme and SSU synthesis were all increased by more illumination. The increase in the rate of SSU synthesis was directly proportional to the increase in translatable mRNA coding for SSU. In addition, the proportion of this mRNA activity in relation to the total mRNA activity almost coincided with that of SSU synthesis in relation to total soluble protein synthesis after induction by light. These results (Sasaki et

al.) suggest that light-induced increase of RuBPC-ase is controlled by the mRNA level for the SSU, which in turn is modulated by a nuclear-transcriptional process or by a light-evoked stabilization of mRNA. During induction the pool of SSU increased with illumination time, in proportion to the total amount of SSU synthesis. The rate of RuBPC-ase synthesis was proportional to the rate of SSU synthesis. These results imply that the induction, which requires the co-operation of nuclear and chloroplast DNA, is regulated by the SSU mRNA level rather than by a cytoplasmic translational process, transportation of the precursor, or assembly of the subunits.

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CHAPTER 7.

GENERAL DISCUSSION AND CONCLUDING REMARKS

7.1. CONTRIBUTIONS OF THE PRESENT SERIES OF INVESTIGATIONS

The present investigations produced some results which should add to our understanding of the chemical composition, genetics and synthesis of subunits of RuBPC-ase. The level of essential amino acids of RuBPC-ase from Medicago leaf tissue was found to be greater than the level of recommended requirements for human nutrition, with the exception of methionine, which was slightly below the required amount. The amino acid composition of RuBPC-ase or 'cytoplasmic' proteins was nutritionally superior to that found for seed storage proteins. Since the RuBPC-ase can comprise as much as 57% of the total leaf protein in Medicago (Chapter 3.2.1.i), this balanced amino acid composition is nutritionally significant.

Isoelectric focusing experiments, using a modified apparatus and a rapid preparation procedure, showed one LSU and two or three SSU polypeptides to be present in the alkaline region of the gel. This was the first evidence that "alkaline" SSU polypeptides occur in plant species. In contrast to my results, Chen et al. (1977) reported that M. sativa had three LSU's and one "acidic" SSU polypeptide. Isoelectric focusing of RuBPC-ase of Spinacea and three Nicotiana species also showed a single LSU polypeptide (Chapter 4.2.8 and 4.2.9) although Chen et al. (1977) claimed that all plant species so far investigated had three LSU polypeptides. It is not unreasonable now to assume that a single LSU is the general rule in the plant kingdom. It was found that the HR genotype had two SSU and the MF genotype of Medicago had three SSU polypeptides, with different pI values (Chapter 4.2.4). Preparation of samples was carried out

without S-carboxymethylation, which is known to affect the number of SSU polypeptides (Chapter 4.3.). S-carboxymethylation in Spinacea resulted in the appearance of two SSU polypeptides instead of one. In the MF genotype of Medicago, S-carboxymethylation showed two SSU polypeptides, as compared to three SSU's for samples not so treated. It appears that S-carboxymethylation does induce anomalous band formation on isoelectric focusing gels.

The selection experiments (Chapter 5.2.1) for "high" protein content increased both RuBPC-ase and 'cytoplasmic' protein content in Medicago. This is the first experimental evidence that RuBPC-ase levels can be increased by selection without doubling the number of chromosomes. It was found that the increased synthesis of both RuBPC-ase and 'cytoplasmic' proteins is mainly under nuclear DNA control (Chapter 5.2.1). A correlation was found between the level of RuBPC-ase and the area of chloroplasts, the organelles where LSU polypeptides are known to be synthesised. These findings also have not been reported previously.

The ratio of LSU to SSU polypeptides was found to be in the expected 4:1 molar proportion, not only in established cultivars, but also in selected genotypes under normal environmental conditions. Growth of lucerne plants at temperatures above 30°C reduced the synthesis of both LSU and SSU polypeptides, but the decrease was greater in the case of the LSU. Genotype x temperature interactions were also demonstrated. This effect of high temperature was different from that in rye (Secale cereale L.), as reported by Feierabend and Wildner (1978). The high temperature induced a deficiency of 70S ribosomes in rye, so that the LSU polypeptide was absent while the SSU polypeptide was still synthesised, and no genotype x temperature interaction was demonstrated. Low daylight

intensity also had a marked effect on LSU and SSU synthesis in Medicago. Again the reduction of synthesis of LSU was greater than that of SSU polypeptides. The genotype x light intensity interaction was significant. Tobin and Suttie (1980) also found an effect of reduced light intensity on LSU and SSU polypeptide synthesis in Lemna gibba, using the incorporation of ^{35}S-methionine as a tracer for protein synthesis but, as the amino acid composition of the subunits was unknown, the relative synthesis of the subunits could not be reliably compared. They also failed to demonstrate genotype x light intensity interactions.

In summary, the present investigations have extended our knowledge of variability, genetic control, and the effect of environmental factors on the synthesis of subunits of RuBPC-ase in Medicago.

7.2. CO-ORDINATED SYNTHESIS, DECLINE OF CO-ORDINATED SYNTHESIS, AND UNCOUPLING OF CO-ORDINATED SYNTHESIS OF RuBPC-ase, LSU AND SSU POLYPEPTIDES

The molar ratio of LSU to SSU polypeptides of established genotypes (Chapter 6.2.2.i - 6.2.2.iii) was close to 4:1, under normal temperature and light intensity conditions. Several environmental factors, namely high temperature (Chapter 6.2.3.) and light intensity (Chapter 6.2.4) reduced LSU polypeptides and the ratio of LSU to SSU of the HP genotype of Medicago.

There are three hypotheses which can be useful in explaining the synthesis of RuBPC-ase and its subunits, as follows: co-ordinated synthesis, co-ordinated decline, and uncoupling of co-ordinated synthesis of the subunits.

Co-ordinated synthesis of LSU and SSU polypeptides has long been suggested for the assembly of RuBPC-ase and this system would

seem to be most efficient, although other types of regulation may also contribute to the LSU/SSU balance. The amount of the two subunits available in cells can be regulated by the actual synthesis of both subunits, by the control of transportation of SSU polypeptides through the membrane of plastids, or by the control of the degradation process acting on the subunits.

There are several examples in the literature which support the above three hypotheses.

Co-ordinated synthesis of the LSU and SSU of RuBPC-ase was also demonstrated during early cellular development in seven-day-old seedlings of Triticum aestivum var. Maris Dove by Dean and Leech (1982). Synthesis of the two subunits in wheat leaves was tightly co-ordinated during cellular development under normal light conditions. Changes in their synthesis occurred simultaneously, as monitored by analysis of the polypeptide products of in vitro translation of mRNA isolated from the leaves.

Chemical uncoupling of the synthesis of LSU and SSU polypeptides was also demonstrated by Barraclough and Ellis (1979) with isolated pea chloroplasts.

A co-ordinated decline in the synthesis of both subunits of RuBPC-ase in ageing wheat leaves (Triticum aestivum L.) has been demonstrated by Brady (1981). The rate of synthesis of RuBPC-ase in the almost fully expanded second leaf approximated the average for all proteins in wheat. The rate fell sharply relative to the average rate of total protein in the days following the full expansion of the leaf. According to analyses of the subunits in the carboxylase holoprotein there was little difference in the rate of decline between the LSU, synthesised on plastid ribosomes, and the SSU which is synthesised on cytoplasmic ribosomes. A protein fraction smaller than

the carboxylase holoprotein, and reactive to antibodies to the carboxylase protein, was also identified in leaf extracts. This fraction was present in greatest amount in young, expanding leaves. Carboxylase subunits in the low-molecular-weight fractions were not labelled rapidly after pulse-labelling of expanded leaves, and there was no evidence of a continuing high rate of synthesis of non-assembled carboxylase subunits in expanded leaves. The cumulative evidence indicates a coordinated decline, with leaf age, in the synthesis of each of the enzyme subunits.

Biochemical changes occurring during the senescence of wheat leaves (vegetative and flag leaves) include reduction of RuBPC-ase activity and photosynthesis (Camp et al. 1982). Activities of stromal enzymes decreased prior to decline in photochemical activity. In general, total soluble protein and the activity of RuBPC-ase and NADP-triose-phosphate dehydrogenase declined in parallel, but these preceded decline or loss of leaf chlorophyll, leaf photosynthesis, and photosynthetic electron transport activity.

It seems that in genotypes of Medicago co-ordinated synthesis, present under normal environmental conditions, can be upset by stress conditions such as high temperature and low light intensity. This uncoupling of co-ordinated synthesis of subunits of RuBPC-ase would consequently affect photosynthesis and general plant development. The nature of the uncoupling of co-ordinated synthesis of LSU and SSU polypeptides in Medicago could be explained by reduced synthesis of subunits, and not by degradation processes.

It can be concluded that the hypotheses of co-ordinated synthesis and uncoupling of co-ordinated synthesis may explain the stress-induced changes of RuBPC-ase in Medicago.

7.3. NUCLEAR CONTROL OF THE QUANTITY OF RuBPC-ase AND OF OTHER CHLOROPLAST ACTIVITY

The mean protein contents of H x L and L x H hybrid generations (Chapter 5.2.1) were very similar and their values were approximately the means of the parental protein contents. These results indicate that the increased synthesis of both RuBPC-ase and 'cytoplasmic' proteins is mainly under nuclear DNA control. This offers support for nuclear determination of the level of RuBPC-ase as previously demonstrated. Also, selection for high and low protein content (Chapter 5.2.1) produced distinct genotypes in which protein content was correlated well with plastid area, a measure of the size of the chloroplasts.

Nuclear control of development and of other synthetic activities in chloroplasts has been reported elsewhere in the literature.

In Oenothera it was demonstrated in experimental crossing between different species that paleness of variegated leaves in hybrids occurs when the nuclear genes do not "harmonize" with the plastid genes (Schötz, 1970). In crosses of Oenothera spp. it was found that when plastids were in harmony with the hybrid genome, normal green chloroplasts resulted in the hybrid leaves, but when plastids were in disharmony with the genome, they remained pale. This is one basis for variegation in hybrids. Fine structural analysis also showed differences in stacking of thylakoids between the "normal" type, which represented harmonic cooperation between nuclear and plastid genes, and the disharmonic-combination types. However, the intensity of "disharmony" changed with time following germination, due to differential activation of nuclear genes. These hybridization experiments provided some support for the currently accepted concept that development of chloroplasts is governed not only by plastid genes but also by co-operating nuclear genes.

Other evidence for nuclear genetic control of chloroplast development has been obtained from an analysis of mutants of barley (Wettstein et al. 1971; Wettstein, et al. 1974). In barley 198 recessive lethal chloroplast mutants were identified and the mutations were assigned to 86 nuclear gene loci. In other words, many albino mutants showing impairment of chloroplast formation are clearly under Mendelian genetic control. Similar genetic control of chlorophyll synthesis has been shown in the analysis of a mutant of Chlamydomonas (Wang et al. 1975).

Evidence for nuclear control of the protein composition of developing photosynthetic membranes has also been reported (Nielsen, 1973; Nielsen et al. 1974; Nielsen et al. 1975; Wettstein et al. 1971; Wettstein, 1974). In particular, Ridley and Leech (1970) found that the electrophoretic analysis of protein in isolated barley chloroplasts showed characteristic patterns. Several new major classes of membrane proteins could be distinguished during greening of seedlings, and alterations were noted in the mutants. Chloramphenicol and cycloheximide, specific inhibitors of the plastid 70S and cytoplasmic 80S ribosomes, respectively, each had different inhibitory effects on the appearance of these new protein bands. Chloramphenicol selectively reduced a particular peak "B", but cycloheximide inhibited peaks "I" and "Y" proteins to a greater extent. In addition, electrophoretic analysis of the polypeptide composition of thylakoids from lethal nuclear-gene mutants and etioplasts of barley have shown absence, weakening or altering of specific bands in the chloroplast pattern (Høyer-Hansen et al. 1976; Høyer-Hansen and Simpson, 1977; Machold and Høyer-Hansen, 1976).

All the results mentioned above emphasise that nuclear genes are involved in the synthesis of chloroplast membrane proteins.

Several other studies on protein synthesis in chloroplasts, involving amino acid incorporation measurements and selective inhibition of 70S ribosomes (Blair and Ellis, 1973; Boulter, 1972; Eaglesham and Ellis, 1974; Ellis 1974, 1975a, 1975b, 1976, 1977; Joy and Ellis, 1975; Siddell and Ellis 1975) led to the interpretation that synthesis of only relatively few chloroplast proteins is entirely under plastid control. By inference, the remainder are either encoded by nuclear genes and synthesised outside the chloroplast, or else have their synthesis in the chloroplast controlled in some way by nuclear gene products. Chloroplasts are still subject to some direct nuclear control even after they have fully matured. This has been convincingly demonstrated in experiments on Acetabularia (Apel and Schweiger 1972; Kloppstech and Schweiger, 1973a,b). Various elegant experiments have been performed with this unique alga, involving such manipulations as enucleation, nuclear transplantation and grafting of stalks. Studies on the water-insoluble proteins of Acetabularia chloroplasts clearly showed that, though they were assembled in situ, synthesis was on ribosomes derived from the nucleus. Both 70S and 80S ribosomes were found in these studies to be present in the chloroplasts, and nuclear transplantation experiments confirmed that some chloroplast ribosomal proteins are coded by nuclear genes (Kloppstech and Schweiger, 1973a,b).

The examples quoted above, though not directly concerned with RuBPC-ase synthesis, nevertheless support the notion that the level of synthesis of the RuBPC-ase enzyme of chloroplasts could well be determined as much by nuclear factors as by chloroplasts factors. Certainly the direct involvement of the nuclear genome provides the most ready explanation of the results of the selection experiments where increased content of RuBPC-ase in 'high' protein selections was achieved without doubling the chromosomes.

7.4. VARIABILITY OF AMINO ACID SEQUENCE OF THE SSU POLYPEPTIDE

The present investigations demonstrated differences in numbers and pI values of SSU polypeptides (Chapter 4.2.4.) and also differences in amino acid composition of SSU polypeptides of Medicago species (Chapter 4.2.5).

Allelic polymorphism has been observed previously in a comparison of the N-terminal amino acid sequences (110 to 120 amino acids) of the SSU in Oenothera biennis, barley, pea, bean, and tobacco (review by Wettstein et al., 1978). Likewise, polymorphism was apparent at the C-terminal end as determined by carboxypeptidase-A digestion (Sugiyama and Akazawa 1970; Strøback, Gibbons, Haslett, Boulter and Wildman 1976, Poulsen 1977). The following three sequences illustrate such differences at a generic level:

Spinach: - Phe-Leu-Thr-Tyr-C00H

Tobacco: - Thr Val Leu Tyr C00H

Barley : - Leu-Tyr-Phe Val-Asn-Ala C00H

The four additional amino acids at the C-terminal end of the SSU in barley are consistent with a slightly higher molecular weight as compared to the SSU of spinach and tobacco.

The complete amino acid sequence of the 120 amino acids of the SSU of RuBPC-ase from spinach has been determined by Martin (1979). Haslett et al. (1976) investigated the amino acid sequence of the SSU of RuBPC-ase from Pisum sativum and Vicia faba. Pea and broad bean sequences were very similar, with only two differences in the first 25 positions. These occurred at position 17, where pea had tryptophan and bean had tyrosine, and at position 23 where pea had proline and bean had glutamine.

The analyses of amino acid sequences of the above listed species showed marked differences. As the isoelectric focusing patterns of the SSU polypeptides of Medicago are different, it is not unreasonable to assume that these differences would be reflected in SSU polypeptides of Medicago as well. It would be interesting to establish the extent of changes in amino acid sequences among closely related Medicago species.

7.5. CONSERVED NATURE OF NUCLEIC ACID SEQUENCES FOR LSU AND SSU POLYPEPTIDES

The pI values of LSU polypeptides of HR and MF genotypes of Medicago were identical (Chapter 4.2.4.) but slight differences were noted between the pI of Medicago, Spinacea and Nicotiana (Chapter 4.2.8.) in the present investigations. These results also indicated only minor modification in the LSU polypeptides of different species.

The conserved nature of LSU polypeptides is also reflected in the nucleic acid sequence as determined for other plants. The chloroplast DNA in maize coding for the LSU of RuBPC-ase has been sequenced in its entirety (including the flanking regions) by McIntosh et al. (1980), using a cloned restriction fragment carrying this gene. The nucleotide sequence from the cloned restriction fragment of maize has been compared with the partial amino acid sequences determined for the RuBPC-ase LSU of barley (Poulsen 1979; Poulsen et al. 1979) and spinach (Hartman et al. 1978). Among the 246 amino acid residues which could be compared for maize and barley, eight substitutions were found. The deduced sequence of the 475 amino acids of the spinach LSU showed 10% divergence from that of the maize LSU (McIntosh et al. 1980).

By contrast, when the SSU of RuBPC-ase from pea, spinach, petunia and wheat was investigated by Smith et al. (1982), some

regions of complete homology between the four genera were found, but the most striking finding was a conserved sequence of only 16 amino acids. The sequence conservation of amino acids was 69% in the SSU of RuBPC-ase of pea, spinach, wheat and petunia. The results contrasted with the 90% conservation of amino acid sequence and 84% conservation of nucleotide sequences for the LSU polypeptides of maize and spinach, confirming that LSU was more conserved than SSU.

Although no nucleic acid sequence analyses were carried out in Medicago, the results of amino acid analysis of Medicago are consistent with the LSU having a more conserved nature than the SSU polypeptide.

7.6. GENETIC CONTROL AND EVOLUTION OF RuBPC-ase

The enzyme, RuBPC-ase, consists of 16 subunits, including eight LSU's which are coded for in the chloroplast genome and eight SSU's coded by the nuclear genome in higher plants. In the chloroplast there is a single gene for the LSU polypeptide, but the nucleus contains from 4 to 12 genes coding for the SSU polypeptide (Dunsmuir et al. 1983).

Nucleotide sequence analysis and amino acid sequence analysis of LSU and SSU polypeptides of various plant species have shown the LSU to be more conserved than SSU (as reviewed in Chapter 7.5).

There are two different hypotheses which may explain the differences in diversity and evolution of LSU and SSU genes. They are the mutation hypothesis, and the multigene structure and function hypothesis.

7.6.1. Mutation Hypothesis

It is expected that the LSU polypeptide of RuBPC-ase would be more variable than the SSU polypeptide, because the LSU gene is

larger and therefore subject to more nucleotide changes, but the opposite appears to be true. One explanation is that the LSU gene, even though larger than the SSU gene, is located on a circular DNA molecule which is different from the nuclear chromosomes (which condition the SSU) in several important respects:

a) Repair mechanisms in the chloroplast, if similar to those in prokaryotes (as are the mechanisms of transcription and translation), would be expected to be more efficient than those operating in the nucleus.

b) The chloroplast chromosome has a high copy number i.e., the circular chromosome is present in the chloroplast at any given time as multiple copies.

Since the chloroplast chromosome or DNA molecule occurs as multiple copies, any genes on this chromosome would be present in higher numbers than would the SSU gene or other genes located on the nuclear chromosomes. In the case of the tetraploid Medicago, the SSU gene is present in 16 copies only.

Another possible explanation for the difference in variability of the two subunits could be that the LSU has been shown to contain the active catalytic site of the enzyme. It could therefore be expected that mutations or other genetic changes in LSU genes would be more likely to have a deleterious effect on the enzyme and on competitiveness or survival of the host plant than would mutations of the SSU, because the loss of RuBPC-ase activity would limit the plant due to impaired photosynthetic capacity. This interpretation rests on the fact that mutations affecting critical or vital cellular functions usually disadvantage the plant, leading to greater selection pressure against the mutated LSU genes.

In contrast, mutation in the SSU gene appears less likely to affect critical functions except possibly for minor differences in stability of the enzyme. A large number of amino acid alterations could be tolerated and accumulated without serious consequences to critical enzyme function.

7.6.2. Multigene Structure and Function Hypothesis

The greater variability of SSU polypeptides as compared to LSU polypeptides might more easily be explained by a multigene structure and function hypothesis as enunciated by Campbell (1983a), the main argument of which is summarized below.

Studies of gene structure have disclosed three organisational features that have not been fully integrated in evolutionary theory. Firstly, genes have significant internal structure. Their substructure is elaborate, unique to each gene, and forms the basis for individual gene function. Multigene family organization enormously increases the capacity of DNA to store, process, and express genetic information. It also allows evolution to proceed quite differently than for simple, single-copy genes. For example, when two or ten or even a thousand similar gene copies are tightly linked together, selection cannot operate effectively on mutations in any one copy. The individual gene is "lost in the crowd" and natural selection is able to survey only the adequacy of the family as a whole.

The second realization concerning gene organization is that genes are chemical substrates for various enzymes. The early view was that genes are static units, except for mutation, but now it appears that genes are variable molecules. As such, their structures can be and are, normally altered by the organism. Cells have diverse enzymes (DNA polymerase, restriction endonuclease, repair enzymes,

translocase, recombinase, replicase, gyrase, mutase, etc.) capable of effecting a variety of alterations in DNA structure. These gene-processing enzymes not only alter the structure and expression of gene molecules but are also essential for the way that elaborate genes, such as multigene families, evolve. The multigene families have evolved elaborate control systems called governors (Campbell, 1983b) for regulating their structural alteration by enzymes.

Gene processing enzymes make the genome far more fluid and dynamic than had been imagined earlier (Hunkapiller et al. 1982). They probably are the source of most of the variability important for evolution. This is significant because enzymes are notable for the specificity of the chemical transformations that they catalyse. The gene molecules thus offer deliberately patterned variation for natural selection.

A third, more sophisticated realization of modern genetics is that genes are "aware" of their environment. Complex genes include sensory devices to bring relevant information to their DNA molecules. Jacob and Monod (1961) presented a model for the basic structure of an "operon", a common type of sensory device among bacterial genes. This system allows a gene for a particular enzyme to be expressed only when substrate for the enzyme is present. Monod (1957) found that the presence of substrate "induces" the expression of the gene (Monod, 1957). Repressor systems are useful (i.e. increase fitness) because they allow bacteria to make enzymes only when appropriate.

Repressors are sensory devices of remarkable versatility, considering their molecular simplicity. In theory, a repressor could evolve to inform a gene about any ligand or condition able to affect the quaternary structure of a protein. While sensors still are best understood in prokaryotes they also are integral components of eukaryotic control systems, such as multigene family governors.

As the mutation theory does not give a wholly satisfactory explanation of genetic variation of SSU polypeptide of RuBPC-ase, the evolution of structure and function of RuBPC-ase of Medicago might be considered in the light of the new evolutionary theory of Campbell (1983a). This theory predicted that a "multigene family" enormously increases the capacity of DNA to process and express genetic information, in contrast to a single-gene system. Investigations of quantitative genetics indicated that multigene variation is more important than single gene variation in Medicago (see Daday et al., 1977 in Chapter 1.12). The LSU polypeptide of RuBPC-ase is under single-gene control and very little change has occurred in nucleic acid sequence during the evolution of plant species. In contrast, the SSU polypeptides are under multiple-gene control and many changes have occurred during the evolution of plant species.

Gene-processing enzymes can cause significant changes to the gene structure. Although the SSU polypeptides of all other species have acid pI values, the pI values of SSU polypeptides of Medicago are all alkaline. This change could be interpreted as due to the action of gene-processing enzymes.

The fact that complex genes may include sensory devices to bring relevant information to their DNA molecules could be relevant to variation in RuBPC-ase subunits. The synthesis of LSU and SSU polypeptides of HP and MF genotypes at 25/19°C and 35/30°C is markedly different. This may be due to some sensory device which regulates the synthesis of subunit polypeptides under particular temperature conditions.

Evolution of structures with altered function may also apply here. The present investigation showed differences in pI values and amino acid composition of SSU polypeptides of Medicago species, which

in turn indicate differences in nucleic acid sequence. The diversity of synthesis of subunits also suggests that these structural changes may result in functional changes in Medicago. The two Medicago species, M. falcata and M. sativa, evolved during thousands of years in two contrasting environments. M. falcata is indigenous to Siberia where long daylight, low light intensity, periodical drought, cool summers and low winter temperature developed a species with a shorter root system, resistance to drought, and preference for lower light intensity. In contrast, M. sativa cv. HP originated in Peru, where it was grown under Mediterranean-type climate conditions, adapted to high light intensity and a short daylight period and developed a long tap root system to escape the drought. It also has a vigorous growth habit. One can therefore assume that there are numerous multigene changes (apart from those affecting RuBPC-ase) in morphological, physiological, or competitive characters of the two species which have resulted in contrasting phenotypic expression.

7.7. FUTURE RESEARCH

Questions arising from the present study have been raised in the course of the Discussion. Those that seem most deserving of further investigation concern the significance of the apparent variations in amino acid composition of SSU in Medicago species. Do they affect only the structural characteristics of the RuBPC-ase protein or do they affect the enzyme function in any significant way? If function is affected, as evidenced by differences in photosynthetic activity, are there also differences in the responsiveness to varying environmental conditions? Finally, given that photosynthetic efficiency of Medicago species can be shown to differ, do such differences influence the fitness value of the genotypes more importantly than other factors, including other complex multigene genotypes? In short, further

investigations should be aimed at evaluating the adaptive significance of RuBPC-ase characteristics in the evolution of plants.

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